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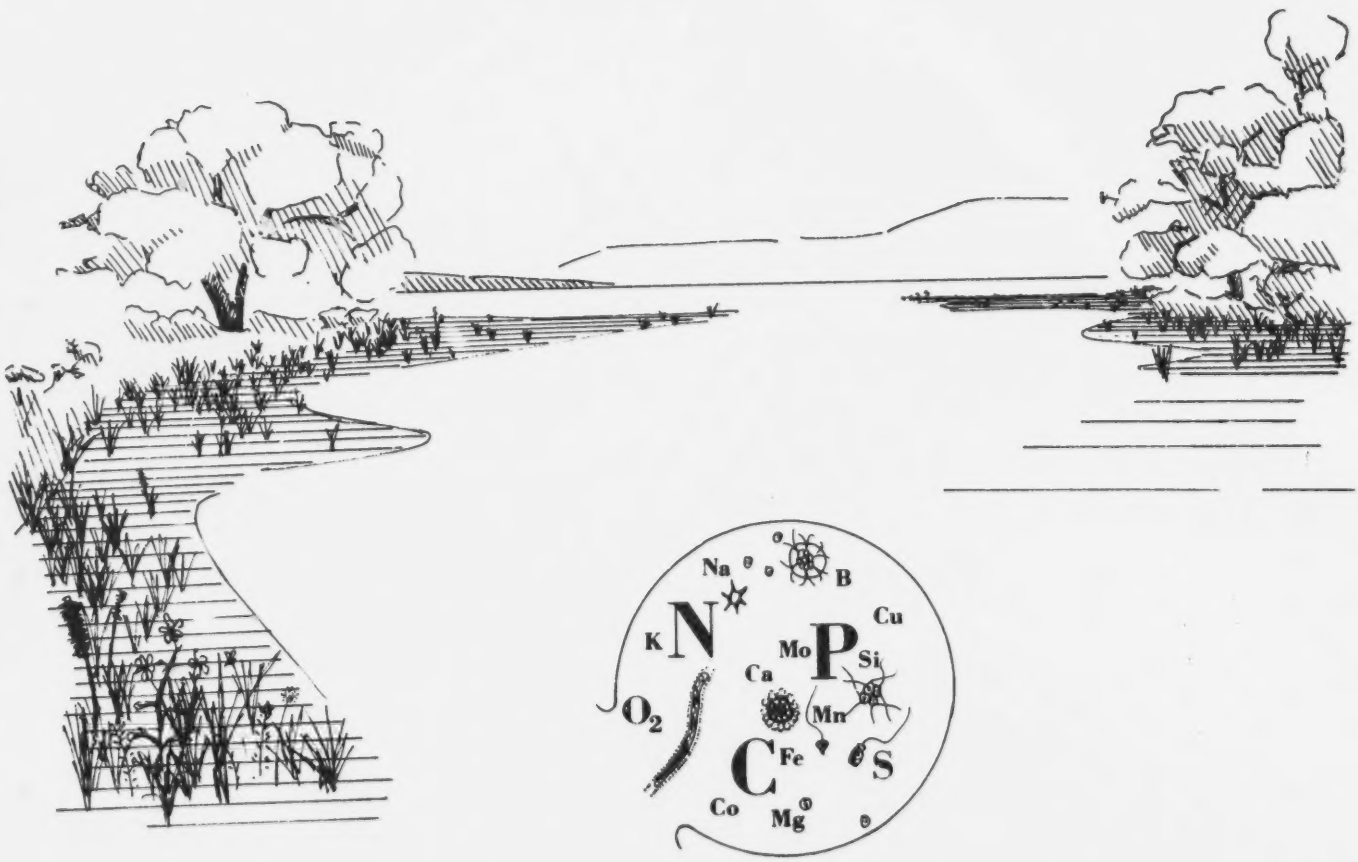

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~~Evaluation of~~ bioassays for nutrient availability were evaluated to define conditions and limits under which each method can give meaningful results. The biological availability of algal nutrients in a water sample and the algal response to changes in the growth-limiting nutrient were measured. Factors other than insolubility prevent the nitrogen or phosphorus of certain samples of aerobic lake muds from being readily available for algal growth; P-limited Spirogyra has been found growing through mud layers containing 0.1% total phosphorus. The facts, that live algae and aquatic weeds do not share their adequate or surplus nutrients with nutrient-limited algae and that lake muds do not provide readily available nitrogen or phosphorus, indicate that once lake waters are stripped of available nutrients by plant production, further plant production will depend upon nutrients from continuous sources, such as wastewater effluents. Phosphorus-starved cells of Anabaena rapidly increase their capacity to reduce acetylene to ethylene when they receive phosphorus. This response may be used as a bioassay for detecting available phosphorus in aquatic ecosystems. Sensitivity of the method compares favorably with conventional methods for measuring dissolved orthophosphate, and has the advantage that it measures available phosphorus. (Jones-Wisconsin)

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NUTRIENT SOURCES FOR ALGAE

AND THEIR CONTROL

By

Dr. George P. Fitzgerald, Principal Investigator
Water Resources Center
University of Wisconsin
Madison, Wisconsin 53706

for the

Environmental Protection Agency

Project #16010 EHR

August 1971

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ABSTRACT

Critical evaluations are presented of bioassays for nutrient availability. The biological availability of any required algal nutrient in a sample of water can be determined by growth experiments requiring 2 weeks incubation. In addition, relatively short-term tests can be carried out measuring changes in certain enzymatic activities or chemical fractions which have been shown to reflect meaningful nutritional changes. The latter types of tests have been useful in evaluating the nutritional status of *in situ* algae. The selection of the type of bioassays for particular purposes can be made from the data presented as to what information can be obtained, the length of time required, and the range of sensitivity of the bioassays. Examples are presented of ecologically important questions which have been answered by the different bioassays.

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SUMMARY AND CONCLUSIONS

A critical evaluation of bioassays for nutrient availability was made in order to define the conditions and limits under which each method can give meaningful results. The biological availability of algal nutrients in a sample of water and the response to changes in the growth-limiting nutrient were measured by the following tests:

The growth attained by selected algae was measured in spiked and untreated samples (as much as 2 to 3 weeks of incubation required).

Available N of the sample was calculated from NH_4-N absorption rates after incubation with N-limited algae (1 or 2 days incubation required).

Available P of the sample was calculated from increases in extractable PO_4-P after incubation with P-limited algae (1 or 2 days incubation required) and from increases in the rate of C_2H_2 reduction by P-limited N_2-fixing algae (1/2 hour incubation required).

To determine if various sources of nutrient are capable of supplying adequate amounts of N or P for *in situ* algae or aquatic weeds the following information was used:

N-limited algae or aquatic weeds will absorb NH_4-N in the dark at rates greater than 15 µg NH_4-N/10 mg/hour.

N_2-fixing algae grown in environments with surplus available fixed N (NH_4+NO_3) will have relatively few heterocysts and low N_2 fixation or C_2H_2 reduction rates.

P-limited algae or aquatic weeds will have less than 0.08 mg extractable PO_4-P/100 mg plant material and have relatively high alkaline phosphatase activities.

P-limited N_2-fixing algae will respond to incubation with added PO_4-P by having increased rates of C_2H_2 reduction.

The following is a summary of limnological facts recently corroborated by bioassay analyses:

The nutritional status of certain species of algae can vary from lake to lake, or even from different areas or depths in the same lake, on the same sampling date; subsurface samples of planktonic algae have been shown to have surplus N or P at times when the same species in surface waters were N or P limited.

Lake Mendota algae contain surplus N and P in spring, can become N or P limited (at the same time or independently) during summer, and again have surplus N and P after the fall overturn. This pattern may represent the changes that take place in similar eutrophic lakes with spring and fall overturns.

Rain can be a significant source of available N to algae in surface waters in the Madison lakes. Less dramatic increases in available P were also associated with certain rains.

Filamentous green algae, such as Cladophora sp., and aquatic weeds that have been in an environment containing surplus N for a week or more are usually visibly coated with epiphytes (have a brown appearance).

Under certain laboratory conditions, solubility was shown not to be a limiting factor in the nutrition of algae because the equilibrium between soluble and insoluble nutrients allowed algae to obtain sufficient amounts of nutrients which were present in an "insoluble" form.

Factors other than insolubility prevent the N or P of certain samples of aerobic lake muds from being readily available for the growth of algae; P-limited Spirogyra sp. have been found growing through layers of muds with 0.1% total P content.

The nutrients of live algae and aquatic weeds are not effectively available to other plants even when nutrient-limited plants are mixed with plants containing surplus nutrients. However, when plants containing surplus nutrients are killed their nutrients become available for nutrient-limited plants. This points out a distinct disadvantage of chemical treatment as a means of controlling undesirable growths of aquatic weeds because weeds killed by chemical treatment are likely to release much of their nutrients to the lake water in forms available for the growth of algae.

NUTRIENT SOURCES FOR ALGAE

INTRODUCTION

Preliminary evaluations of water pollution control projects for the control of obnoxious growths of algae will depend upon the relative sources of nutrients: those which can be controlled *versus* those which cannot. Through the use of bioassay techniques developed in this laboratory for nutritional studies of problem-causing algae it is proposed to study the sources of nutrients available to algae and evaluate possible means of limiting these available sources. The factors that control the absorption and utilization of nitrogen, phosphorus, and possible other nutrients will be studied by the use of techniques developed for determining if algae have surplus quantities of certain nutrients or are limited by their supply of these nutrients. The main questions to be investigated will be: 1) the sources of nitrogen and phosphorus which can serve as available nutrients for algae; 2) the factors affecting the utilization of various nutrients under the conditions existing in surface *versus* bottom water of lakes; 3) whether the solubility of nitrogen, phosphorus, and iron compounds in the bottom of lakes is a factor in the nutrition of algae (precipitated *versus* soluble nutrients); 4) assuming that phosphorus is an important nutrient for algae in lakes fertilized by sewages or treated sewages, if the phosphorus of detergents were replaced by organic chemicals such as nitrilotriacetate or E.D.T.A., what effect might this have on the practical supply of phosphorus from sewages; and 5) what effect would such a replacement have on the availability of other nutrients and on the copper sulphate treatments used for the control of algae in lakes and reservoirs.

Bioassays can be used to evaluate the amount of a particular nutrient or nutrients available to algae or aquatic weeds in a water sample or to assess the nutritional status of *in situ* plants. Bioassays for any required plant nutrient can be carried out by growth experiments in the laboratory using selected species of algae. In addition, relatively short-term tests can be carried out by measuring changes in certain enzymatic activities or chemical fractions which have been shown to reflect meaningful nutritional changes. An evaluation of the N or P nutritional status of *in situ* algae or aquatic weeds at any particular time can be made by measuring the NH_4-N absorption rates in the dark, relative amounts of PO_4-P extracted and alkaline phosphatase activities, or N_2-fixation rates by blue-green algae. Bioassays of water samples demonstrate the level of available nutrients whereas bioassays with *in situ* plants demonstrate

whether the environment has supplied nutrients to only a limited extent or if there were surplus quantities available. The latter tests also will indicate transitory changes in nutrient sources that might have taken place between sampling dates, such as the effects of slugs of nutrients which might not be detected without continuous monitoring.

Through the use of the bioassays developed and evaluated by laboratory studies several practical evaluations of *in situ* nutrient sources have been made. The results of these tests have suggested methods that could be used to control certain forms of eutrophication of natural waters or the inadvisability of further pursuit of certain approaches to eutrophication control.

MATERIALS AND METHODS

Analysis of Nutrient Content by Growth

The value of measuring the growth of algae in water samples is that differentiation can be made between the total nutrient content of water samples as obtained by chemical analyses and the nutrients that are available to support the growth of algae under certain circumstances. The concentration of any nutrient required for the growth of algae can be determined by measuring the growth attained by selected algal species in dilutions of the water sample or after suitable spikes of other nutrients are added. Standard techniques for this type of bioassay are being developed (Maloney, 1970). Growth experiments often require two or three weeks of incubation, but preliminary results can sometimes be detected after only one or two days. Since long incubation periods may allow an original source of a potential nutrient to degrade to an available form (such as a polyphosphate compound degrading to orthophosphate), it is sometimes desirable to expose the algae for relatively short periods of time to the potential nutrient and then transfer them to another media to grow on the absorbed nutrient (Fitzgerald, 1970a).

Some factors that can influence the results of nutritional tests are the type, source, and amount of algae used and the nutrients carried over when the algae are added to the test media. The algae to be used must be readily available and must respond to the nutrients of interest. In addition, the selected species should represent problem-causing species since not all algae may respond alike to the same environment. There is frequent controversy as to whether one should use the natural flora of a water sample for nutrient bioassays or a standardized culture that may not be related to a particular flora but which has been shown to respond similarly to various nutrients. As pointed out, the alga to be used must be available when you want to test the water; if the tests are to be run on samples taken at spring overturn of a lake, the *in situ* algae will probably not be the same as those that cause problems in mid-summer. If the samples must be collected and stored until a convenient time is found for the bioassays, there is a good chance the original flora will no longer be present. It has been pointed out by several workers that the algal species composition of *in situ* tests frequently changed when samples were confined for a few days time, such as the replacement of phytoplankton by

epiphytic types of algae that had become attached to the walls of the container. Therefore, except for certain specific studies, the use of selected and tested species of algae is preferred over the use of *in situ* algae for growth bioassays.

Since algae are known to be able to concentrate certain nutrients in excess of their present needs when they are grown in media with surplus nutrients, this factor must be taken into account in selecting culture media and the amount of algae used. The effect the media can have on nutrition tests was demonstrated by Gerloff and Skoog (1954). They showed that the planktonic blue-green alga, *Microcystis aeruginosa* (Wis 1036), cultured in a medium with low N (6.8 mg N/L) would not grow when transferred to a medium lacking N, but *Microcystis* from a medium with surplus N for maximum growth (27 mg N/L) was able to increase twofold in a medium lacking N. They also showed that *Microcystis* from a medium with excessive P (1.8 mg P/L) could increase fourfold when transferred to a medium lacking P. Using the green algae, *Chlorella pyrenoidosa* (Wis 2005) and *Selenastrum capricornutum* (PAAP), and the blue-green algae, *Microcystis* and *Anabaena flos aquae* (Ind 1444), a series of 30 experiments have shown that there was no significant further growth in media lacking N or P with algal inoculation levels of from 10,000 to 1 million cells/ml from the relatively dilute PAAP medium (4.7 mg N/L and 0.2 mg P/L). Thus, if algae are cultured in relatively dilute media, the amount of growth in subsequent media will be dependent upon the N or P of the latter media regardless of inoculum size. However, if algae were precultured in more concentrated media, such as Allen's (1952) (178 mg N/L and 45 mg P/L) or Gorham's (1958) (82 mg N/L and 7 mg P/L), *Selenastrum* could increase two- to threefold in N-free media and increase fourfold in P-free media. The growth that occurs in N-free or P-free media is due to surplus nutrients (luxury consumption) inside the algal cells. Therefore, the amount of algae added to test media from more concentrated media should be low enough so the excess nutrients in the cells would be insignificant compared to the nutrients in the test media. Statistical data have shown that the lowest concentration of algae that could be readily measured by cell counts using a haemocytometer was 100,000 cells/ml (100 cells, $\pm$ 20, in 175 microscopic fields), so inoculations at levels less than 100,000 cells/ml should be used when cells from relatively concentrated media are used. The carry-over of extracellular nutrients from the preculture medium can be minimized by washing cells in media lacking the nutrients of interest, such as by centrifuging and resuspending in distilled water containing 50 mg/L of $NaHCO_3$.

The sensitivity of growth tests carried out with these precautions can be evaluated by the minimum detectable concentrations of the nutrients of interest. By washing the green alga, Selenastrum, from any nutrient medium and inoculating to a concentration of 50,000 cells/ml, concentrations of various nutrients as low as those presented in Table 1 will consistently yield increases in the measurements used. By using more sensitive measurements than those indicated, lower concentrations of nutrients could be detected.

In order to evaluate the reproducibility of data on the relationship between algal growth measurements and nutrient levels in natural waters, the amount of growth attained by Selenastrum was measured by 3 to 5 tests in each of 8 samples of water from 3 lakes in the Madison area which were preserved by autoclaving. Concentrations of available N in 5 separate tests of a Lake Mendota surface water sample, collected and preserved on August 17 and assayed during December, 1970, were 0.15, 0.17, 0.20, 0.20, and 0.25 mg N/L. This degree of reproducibility is typical for bioassays. The level of available N in [illegible] surface water samples collected in mid-summer of 1970 was about 0.2 mg N/L, whereas a hypolimnion sample contained 1.2 mg N/L, and a surface sample collected in December contained 0.5 mg N/L. These values correlate well with levels of NH_4+NO_3 obtained by chemical analyses of similar samples.

In general, growth of algae in water samples or with potential nutrient sources will be correlated with the growth attained by the same inoculum under conditions of known nutrient content: such as growth in 0, 1x, 2x, 4x, 8x mg/L. Growth in the absence of the nutrient of interest will indicate growth attainable with the nutrients contributed by the algae and the techniques of culturing and handling. The concentration of nutrients used to establish standard curves will be dependent upon the particular nutrient of interest, but should represent as wide a range of nutrients as will have a significant influence on algal growth. The growth of algae in test samples, or dilutions of test samples if concentrated sources of nutrients are suspected, and growth attained with known concentrations of nutrients is used to estimate the amount of nutrients available in the test sample under the conditions of the test.

The availability of different forms of nutrients can also be evaluated by growth experiments. Certain relatively insoluble sources of nutrients, such as iron-phosphorus compounds and teeth for P, hair for N, iron pyrites for iron, and marble for carbon, were found to be readily used by algae, whereas other sources, such as the N or P of

Table 1. Minimum Detectable Concentrations (mg/L) of Nutrients by Growth of Selenastrum When Algal Growth Is Measured by Different Techniques

Nutrient	Absorbance (1 cm, 750 mμ)	Cell Counts (Haemocytometer)	Fluorometry (Chlorophyll a)
N	0.2	0.1	0.1
P	0.02	0.01	0.01
Mg	0.02	0.01	0.01
S	0.02	0.01	0.01
Fe	0.002	0.001	0.001

aerobic lake muds or the N or P contained in other live plants, were found to be relatively unavailable.

Evaluations of the availability of sources of nutrients also can be carried out using rooted aquatic weeds, such as Lemna minor, a common "duckweed." Increases in frond numbers or dry weight can be readily followed during incubation periods of 2 to 3 weeks or after exposure tests of a few hours and then subculture to N- or P-free media for growth on sorbed nutrients. Concentrations as low as 0.25 mg N/L or 0.05 mg P/L will result in at least twofold increases in growth over control (-N or -P) cultures. Because these plants have roots, they can be used to differentiate between nutrient sources available to algae and those that are available to rooted aquatic weeds.

Extractive and Enzymatic Analyses for P

An extractive procedure can be used to differentiate between algae which have surplus or stored P and those that are P-limited. Algae and aquatic weeds containing adequate P will release more than 0.08 mg PO_4-P/100 mg (dry weight) of plant material when extracted in a boiling water bath for 1 hr. Therefore, this extractive procedure can be used to measure the P-nutritional status of algae or aquatic weeds and to follow the effects of environmental changes that might influence the P-nutrition of plants (Fitzgerald and Nelson, 1966).

The PO_4-P in algal extracts was analyzed by the stannous chloride method (Am. Public Health Assoc., 1965). All analyses are reported on a dry weight basis. Dry weight of planktonic algae was measured on filtered samples dried in tared vessels. Measurements of the dry weight of plants that could not be sampled by aliquot, such as Cladophora or Myriophyllum, were made with the actual samples used by filtering and drying them after the PO_4-P extraction or enzymatic tests were completed. Dry weights of samples after extraction with boiling water are not equal to the total dry weight of the original sample but are accurate enough for comparative purposes.

The extraction procedure for surplus phosphorus involved placing 10-80 mg of washed [Gorham's minus P(-P) medium] plant material into 40 ml of Gorham's medium (minus P source; pH 7), extracting in a boiling water bath for 60 min, centrifuging or otherwise removing plant material, and analyzing the supernatant liquid for orthophosphate. Alternative methods using 5 min direct boiling or auto-

claving techniques have not given as complete an extraction as 1 hr in boiling water baths but could be of comparative use. Any extraction method dependent on killing the plant tissue and allowing the PO_4-P to leach to the supernatant liquid would probably be sufficient as long as results could be correlated (Fitzgerald and Faust, 1967). The PO_4-P in the extracts was calculated as mg P/100 mg (dry wt) plant material.

Alkaline phosphatase activity was determined by suspending 1-20 mg of washed plant material in 32 ml of Gorham's (minus P) medium, adding 4 ml of buffer solution (1 M Tris, 0.01 M MgCl, adjusted to pH 8.5 with acetic acid) and 4 ml of p-nitrophenylphosphate solution (30 mg/100 ml), and incubating at 35-37C. The relative activity was measured after 0.25-2 hr by centrifuging 10-ml samples with 10 mg of orthophosphate-P in 0.5 ml (to stop further enzyme activity) and measuring the optical density of the clear supernatant liquid at 395 mμ. An alternative measurement of alkaline phosphatase activity uses commercial enzymatic tablets, one brand of which (Phosphatabs-Alkaline Quantitative, Warner-Chilcott Labs, Morris Plains, N.J.) uses phenolphthalein phosphate and necessary cofactors. Data so obtained are comparable to results with p-nitrophenylphosphate as substrate. Activity was recorded as units of enzyme/mg (dry wt) of plant material in 40 ml. One unit of alkaline phosphatase is defined as the amount of enzyme liberating 1 mμmole of nitrophenol/hr under the prescribed conditions.

The use of the extractive procedure in laboratory studies of the availability of P sources has indicated that 10 mg of either the green alga, Cladophora sp, or leaves of the aquatic weed, Myriophyllum sp, could detect P concentrations as low as 0.04 mg P/L. By using P-limited Cladophora sp from Lake Wingra it was shown that 1% or less of the P of various lake muds was available to the alga when tested under aerobic conditions in the laboratory (Fitzgerald, 1970b).

It has been found that plants that are P-limited will have 25 times as much alkaline phosphatase activity as plants grown with surplus P. Analysis of alkaline phosphatase activity can thus be used to confirm that plants with low extractable PO_4-P levels are alive, but P-limited. The two procedures have been used together to detect long-term nutritional changes, such as seasonal changes in the availability of P in lake waters, or recent additions of available P. Plants that have only recently been exposed to increased available P supplies have higher extractable

PO_4-P but also have relatively high alkaline phosphatase activities because the alkaline phosphomonoesterase content is only lowered by dilution by growth of the cell under adequate P conditions. During the period of July 22 to 31, 1968, the Cladophora sp along the shore of Lake Mendota appeared to be P-limited in that very little PO_4-P could be extracted (0.03 to 0.07 mg/100 mg algae) and relatively high alkaline phosphatase activities were recorded (about 1,000 units/mg algae). There was a sudden increase in extractable PO_4-P from the Cladophora during the period of August 5 to 7, 1968 (probably associated with the 1.4 inches of rain occurring then), the values going to 0.12 mg PO_4-P/100 mg on August 6, but the algae still had 1,500 units of alkaline phosphatase/mg. With growth under surplus P conditions (probably associated with a lack of competition because there were few phytoplankton present; Secchi depth of 2.5 meters), by August 29 the Cladophora had 0.17 mg of extractable PO_4-P/100 mg, but only 90 units of alkaline phosphatase/mg.

Verification of these tests can be accomplished by the contrasting results obtained by the PO_4-P extraction method and alkaline phosphatase measurements with in situ algae, except in cases where recent sources of P were absorbed by the plant material. In addition, plants that appear to be P-limited can be kept for several days in media containing relatively high levels of P, such as Gorham's culture medium. If these plants then absorb P and had been able to absorb P if it had been available in their former environment, they will respond by increases in extractable PO_4-P after incubation in an environment with known available P. Plants that appear to have surplus P can be incubated for a week or more in culture media lacking only P to show that if less P had been available in their former environment they would have lower extractable PO_4-P values and higher alkaline phosphatase activities.

Rate of Ammonia Absorption for N Nutrition

Plants that are limited by the supply of available N are able to absorb ammonia (NH_4-N) in the dark 4 to 5 times more rapidly than plants with adequate or surplus N (Fitzgerald, 1968). Thus, the effect of changes in the environmental supply of N to in situ algae or aquatic weeds can be followed as well as an evaluation made of different sources of N in laboratory experiments by NH_4-N absorption measurements.

Comparative rates of NH_4-N absorption in the dark were used

to differentitate between plant material from cultures containing surplus nitrogen and those whose growth was limited by the available nitrogen. From 5 to 20 mg (dry wt) of plant material was washed in nitrogen-free medium, placed in 10 to 30 ml of Gorham's medium (minus N), and 0.1 mg NH_4-N was added. After 1-hr incubation at 25 ± 2C in the dark, the NH_4-N content of the supernatant liquid was compared to controls without plant material. NH_4-N was analyzed by direct Nesslerization (APHA, 1965). Results were calculated as µg N absorbed/(10 mg dry wt x hr). If the plant material settled during incubation, occasional mixing will be required to prevent local depletion of NH_4-N and erroneous rate results. Without such a depletion, the rates of NH_4-N absorption by samples with fourfold differences in weight will be equivalent on a dry weight basis.

The use of the rate of NH_4-N absorption tests for evaluating the concentration of available nitrogen in water samples has not been investigated in detail. Therefore, the minimum detectable levels of available N by this technique are not known.

Verification of results of this test can be accomplished by incubating plants in relatively high levels of available N or in media lacking only N and recording the changes caused by such treatments. If the plants had been healthy and capable of change in their former environments, they would respond after the incubation treatments.

An interesting correlation has been made between the N nutrition of filamentous algae or aquatic weeds and the growth of epiphytic algae. Observations in the field and in controlled laboratory tests have indicated that plants growing for a week or more in the presence of surplus N usually become coated with epiphytic algae. Therefore, the presence of a dense coating of epiphytes on algae can be used as an indication that the algae have had surplus N available in their immediate past history (Fitzgerald, 1969).

Nitrogen Fixation Rates Related to Sources of N and P

It has recently been shown that the capacity of blue-green algae and other plants to fix N_2 could be followed by measuring the rate of acetylene (C_2H_2) reduction by the same nitrogenase enzymes used to fix N_2 (Stewart *et al*, 1967). The ease with which the rate of reduction of C_2H_2 to ethylene (C_2H_4) can be measured with gas chromatography has made this measurement a useful tool in limnology.

The general assay procedure used was to place about 1 mg (actual dry weight of samples used determined on aliquot samples) of algae in a serum bottle for the direct assay of the C_2H_2 reduction activity or in a volume of test water as a pre-incubation for the absorption of available P before the C_2H_2 reduction assay. The P absorption incubation period can be as short as 30 minutes. The algal suspension is then concentrated by centrifugation to a volume of 1 ml for the C_2H_2 reduction assay. The C_2H_2 reduction measurements were performed in 7-ml-capacity serum bottles fitted with serum stoppers. A 1.0-ml aliquot of test alga was added to each bottle and 1.4 ml of C_2H_2 (purified grade obtained from Matheson Co) was added to the bottle without removing the air. The gas pressure within the bottle then was restored to atmospheric by pricking the serum stopper with a hypodermic needle. This simplified procedure was adopted because experiments showed that it was unnecessary to remove N_2 from the gas phase if sufficient C_2H_2 was added. Acetylene assays were run for 30 min. The experiments were terminated when required by the injection of 0.2 ml of 5N H_2SO_4.

The gas phase was analyzed for ethylene by gas chromatography using a Varian-Aerograph model 600 D gas chromatograph fitted with a hydrogen flame ionization detector and a 9 ft long column of Porapak R. The instrument was run at room temperature and high purity nitrogen gas at a flow rate of approximately 25 ml/min served as carrier gas.

When the availability of P in test samples is to be measured using P-limited algae, all test samles which are being compared should be set up at the same time. For each test water sample (a) there should also be available a control sample (b) in which the algal response in phosphorus-free medium is noted, and a second control (c) comprising the test water sample plus 0.025 mg/liter of phosphorus. The value for (b) serves to show that the bioassay organism is phosphorus starved while the value for (c) shows its ability to respond in the test water when phosphorus is available. The relative response for each test water thus will be: $(c - b)/(a - b)$. By comparing the values for this ratio, the relative abundance of phosphorus in each test sample can be measured.

The quantitative determination of the amount of available phosphorus in a particular water is more complicated, and it is suggested that the following series should be set up for such a measurement: (1) P-free medium, (2) P-free medium + 0.100 mg P/liter, (3) P-free medium + 0.050 mg P/liter, (4) P-free medium + 0.025 mg P/liter, (5) P-free medium + 0.010 mg P/liter, (6) test water sample, (7) test water sample + 0.025 mg P/liter.

Tests (1)-(5) show the response of the alga to available phosphorus in the absence of inhibitory or competitive reactions and can be used to prepare a standard curve which can be used for all tests performed with the same batch of assay organism. The value for test (7) minus test (1) gives the response to 0.025 mg/liter of phosphorus plus the amount of phosphorus in the water sample. The value for test (6) minus test (1) gives the response to available phosphorus in the test water only. Thus the response to 0.025 mg P/liter alone and to the available phosphorus in the test water is obtained. These values are then transferred onto the standard curve to give the quantity of available phosphorus in the test water. Thus, the response of the algae to a series of standards and to two samples of the test water, one with and the other without added phosphorus, is all that is required to determine the level of available phosphorus in a particular water. The same standard curve can be used for all waters tested using the same batch of assay organism. We always have noted a response when phosphorus was added to phosphorus-deficient culture medium. This indicates that a failure to detect acetylene reduction in test water samples with added phosphorus is because of some inhibitory factor in the water. In the presence of total inhibitors the test would not be satisfactory.

Anabaena flos-aquae is a good assay organism, but any fast-growing N_2-fixing blue-green alga presumably could be used. The exact culture medium used is also immaterial as long as it is free of combined nitrogen and the bioassay organism is phosphorus starved and metabolically active at the start of the assay.

Small volumes (25 ml) of test water may be used, but larger volumes are more satisfactory. A volume of 70-200 ml of water is recommended with 6.0 mg dry weight of the assay organism. The volume and amount of algae should be constant in any one test series.

The sensitivity of C_2H_2 reduction assays is such that reproducible measurements can be obtained with 2 to 4 Gloeotrichia colonies per sample. The actual amount of algae used in field work is usually based on the dry weight analyses of samples of concentrated plankton or the total N analyses of the samples used after the C_2H_2 reduction assay. When P-limited Anabaena is used to assay for available P in water samples, as little as 0.01 mg P/L will cause a 100% increase in the C_2H_2 reduction activity of the test alga.

The C_2H_2 reduction assay for nitrogen fixation capacity of field or laboratory algae can be correlated with the rela-

tive numbers of heterocysts (clear cells in which the nitrogenase enzymes seem to be located) and vegetative cells. Algae grown with adequate fixed N (NH_4 or NO_3) cannot fix N_2 nor do they have heterocysts. When N-fixing algae are used to assay for available P, a positive response to added available P serves to indicate the test algae were P-limited, but further evidence can be obtained by measuring the amount of extractable PO_4-P of the algae.

EXPERIMENTAL

Factors Affecting Bioassays by Growth

Bioassays for any plant nutrient can be carried out by growth experiments in the laboratory using selected species of algae. Studies have been made of the influence on the results of nutritional bioassays of some of the factors that can be readily controlled, such as inoculum size, control culture medium (source of standard curves), or source of algae (preculture medium).

A theoretical approach can be used to determine the effect of inoculum size on results of nutrition tests. By using the minimum biomass or equivalent measurement as a basis, the amount of cells to give a significant increase in the measurement can be calculated. Thus, in the case of cell counts using a haemocytometer, in order to have an accuracy of 20%, the lowest reproducible cell count would be about 100,000 cells/ml (100 cells, ± 20, in 175 microscopic fields). This concentration of cells is too dilute to readily measure by absorbance or dry weight, but could readily be measured by fluorometry or with electronic particle counters. The general relationship between the various measurements indicates that an absorbance (1 cm, 750 mμ) of 0.1 is equivalent to approximately 5,000,000 cells/ml and 50 mg, dry weight/L. Therefore, bioassays which do not produce at least 100,000 cells/ml at the final harvest cannot be measured with reasonable accuracy by using the haemocytometer cell count method. Inoculum levels must be such as to bring the number of cells up to a level at harvest time where they can be counted. Only then can a study be made of the effects caused by different preculture media or a determination made of the minimum detectable concentrations of nutrients. If more sensitive biomass measurements are used, lower inoculum levels can be used. However, if algae of different forms, such as unicellular and filamentous forms, are to be compared, measurements are restricted to those methods adaptable to any type of algae. Thus, absorbance and fluorometry can be used under such conditions.

Inasmuch as the bioassays of interest are related to measurements of available nutrients, studies have been made of the effect of different factors on the response of algae to various levels of N, P, Mg, S, and Fe. Since both unicellular algae, Selenastrum, Chlorella, and Microcystis, and the filamentous alga, Anabaena, were employed in the tests, the biomass measurement chosen for use was absorbance (1 cm, 750 nm).

The first series of tests were carried out to demonstrate that the selected algae grown in the relatively dilute PAAP medium and washed in $NaHCO_3$ solution (50 mg/L) could be used to detect the presence of available N, P, Mg, S, and Fe by comparing the growths attained after 7 to 12 days incubation in media lacking the nutrient of interest and with different levels of the nutrient. The results are summarized in Table 2.

It is evident that algae from this dilute medium could be used to detect the nutrients listed. The differences between growths attained in the medium lacking any of the nutrients and in the lowest concentrations of nutrients indicate that, with more sensitive biomass measurements, effects caused by considerably lower concentrations of some of the nutrients could have been detected. However, the purpose of the experiment was to show that these algae could be used to detect available forms of N, P, Mg, S, and Fe when inoculated from PAAP medium at the concentrations used.

Further tests were carried out to determine if five- or tenfold increases in the initial concentration of cells from the relatively dilute PAAP medium would prevent the detection of available forms of N, P, Mg, S, or Fe in PAAP medium. Selenastrum was tested at 10,000 and 100,000 cells per ml, while Microcystis was tested at 50,000 and 250,000 cells per ml. Typical results are summarized in Table 3.

These algae could be used at concentrations of 100,000 or 250,000 cells per ml to detect the differences between PAAP media lacking N, P, Mg, S, or Fe and media with additions of low concentrations of these nutrients.

Since many practical evaluations of available nutrients will not be run in the medium algae are grown in, such as transferring algae from complete PAAP medium to PAAP medium lacking some essential nutrient plus the source of nutrient of interest, tests were carried out in which Selenastrum and Microcystis were grown in PAAP medium and then inoculated in Gorham's medium or Gorham's medium with no or low concentrations of N, P, Mg, S, or Fe. The results of growths attained after 7 to 12 days are summarized in Table 4.

The differences in the growths of these algae in media lacking N, P, Mg, S, and Fe and in media to which low concentrations of these nutrients were added indicate that when algae are transferred from the relatively dilute PAAP medium to the more concentrated Gorham's medium, the availability of the nutrients listed could readily be detected.

Table 2. The Growth of Selected Algae Taken from PAAP Medium and Placed in PAAP Media Containing Different Levels of N, P, Mg, S, and Fe. Growth Recorded as Absorbance (1 cm, 750 nm) after 7 to 12 Days Incubation.

	Growth Attained (Absorbance)			
	Initially 10,000 cells/ml		Initially 50,000 cells/ml	
Test Medium	Selenastrum	Chlorella	Microcystis	Anabaena
Complete	0.18	0.21	0.19	0.18
-N	.005	.04	.02	.20
" + 0.25	.025	.07	.03	.24
" + 0.5	.06	.09	.05	.20
" + 1.	.10	.09	.08	.18
-P	0.0	.005	.01	.01
" + 0.025	.025	.05	.07	.04
" + 0.05	.06	.08	.08	.06
" + 0.1	.08	.10	.20	.09
-Mg	.03	0.0	.02	.01
" + 0.025	.08	.04	.12	.02
" + 0.05	.10	.09	.15	.03
" + 0.1	.13	.09	.14	.06
-S	.02	.02	.02	.03
" + 0.025	.04	.04	.06	.04
" + 0.05	.06	.05	.07	.08
" + 0.1	.08	.07	.10	.09
-Fe	.02	.03	.02	.05
" + 0.001	.06	.04	.03	.07
" + 0.002	.08	.06	.04	.09
" + 0.005	.08	.08	.06	.11

Table 3. The Effect of Different Initial Cell Densities on the Response of Algae from PAAP Medium to Relatively Low Concentrations of N, P, Mg, S, and Fe in PAAP Medium. Growth Attained after 7-12 Days Measured as Absorbance (1 cm, 750 nm).

Test Medium	Growth Attained as Absorbance			
	Selenastrum		Microcystis	
	10,000 cells/ml	100,000 cells/ml	50,000 cells/ml	250,000 cells/ml
Complete	0.13	0.13	0.19	0.27
-N	.005	.015	.015	.03
" + 0.5	.04	.05	.05	.06
-P	0.0	.01	.01	.05
" + 0.05	.06	.07	.08	.22
-Mg	.03	.04	.015	.04
" + 0.05	.10	.11	.15	.12
-S	.02	.02	.02	.02
" + 0.05	.06	.05	.06	.10
-Fe	.01	.02	.015	.06
" + 0.005	.04	.05	.06	.10

Table 4. The Response of Selenastrum and Microcystis Precultured in PAAP Medium to Low Levels of N, P, Mg, S, and Fe in Gorham's Medium. Growth Attained Measured after 7 to 12 Days as Absorbance (1 cm, 750 nm).

Test Medium	Growth Attained as Absorbance	
	Selenastrum 10,000 cells/ml	Microcystis 50,000 cells/ml
Complete	0.38	0.66
-N	.005	.01
" + 0.5	.03	.04
-P	0.0	.015
" + 0.05	.04	.06
-Mg	.10	.01
" + 0.05	.16	.12
-S	.10	.14
" + 0.05	.15	.21
-Fe	.04	.015
" + 0.005	.08	.27

Some concern has been expressed by various authors for the effects on nutritional bioassays of nutrients carried over as surplus stored nutrients inside algae when algae from relatively concentrated media are used as the preculture medium. The four algae, Selenastrum, Chlorella, Microcystis, and Anabaena, were cultured in the relatively concentrated Allen's medium (178 mg N/L and 45 mg P/L) and inoculated into PAAP media lacking N, P, Mg, S, or Fe and with low concentrations of these nutrients in order to determine if such a source of algae would negate the use of the algae for nutritional bioassays. The results with inoculations of 10,000 and 50,000 cells/ml are summarized in Table 5.

When such low initial concentrations of cells are used to detect available sources of N, P, Mg, S, or Fe, algae from the relatively concentrated Allen's medium can be successfully used. Even at an inoculation level of 50,000 cells/ml Microcystis and Anabaena could detect the addition of the nutrients tested.

It was of interest, therefore, to determine the effect increased inoculation densities of algae from the relatively concentrated Allen's medium would have on nutritional bioassays. Selenastrum was cultured in Allen's medium and added to various levels of N, P, Mg, S, or Fe in PAAP medium at initial concentrations of 10,000, 100,000 and 800,000 cells/ml. The results of a series of tests are summarized in Table 6.

There was a logical response to the addition of all nutrients tested when an initial concentration of 10,000 cells/ml was used. Inoculation levels giving 100,000 cells also could detect the addition of N, P, Mg, and S, but the growth in medium lacking Fe was nearly as much as when Fe was added in the case presented. When inoculation levels of Selenastrum from Allen's medium were increased to 800,000 cells/ml, there was a measurable increase in the amount of growth attained in all nutrient solutions lacking one nutrient when compared with the growths at lower cell densities. The Selenastrum, however, did increase over 100% with additions of N and Mg, but not to additions of P, S, and Fe. Furthermore, tests carried out with initial cell densities of one million or more/ml have indicated that all algae tested (Selenastrum, Chlorella and Microcystis) could increase in biomass twofold in media lacking N and fourfold in media lacking P when algae were precultured in media containing surplus quantities of N or P. Therefore, limited amounts of growth of algae can take place with surplus nutrients stored in algal cells from relatively concentrated culture media, but by using inoculation levels of 100,000 cells/ml or less the amounts of nutrients carried over will not prevent the algae from

Table 5. The Response of Algae Precultured in Allen's Medium to Low Levels of N, P, Mg, S, and Fe in PAAP Medium. Growth Attained after 7 to 12 Days Measured as Absorbance (1 cm, 750 nm).

Test Medium	Growth Attained as Absorbance			
	10,000 Cells/ml		50,000 Cells/ml	
	Selenastrum	Chlorella	Microcystis	Anabaena
Complete	0.14	0.13	0.26	0.19
-N	.01	.015	.015	-
" + 0.5	.04	.07	.04	-
-P	.01	.005	.02	.02
" + 0.05	.08	.08	.16	.07
-Mg	.03	.04	.05	.01
" + 0.05	.10	.07	.17	.06
-S	.02	.02	.015	.04
" + 0.05	.05	.10	.08	.08
-Fe	.03	.02	.02	.09
" + 0.005	.10	.05	.17	.14

Table 6. The Effect of Different Initial Cell Densities on the Response of Selenastrum from Allen's Medium to Low Concentrations of N, P, Mg, S, and Fe in PAAP Medium. Growth Attained after 7 to 12 Days Measured as Absorbance (1 cm, 750 nm).

Test Medium	Growth Attained as Absorbance		
	No. Cells 10,000/ml	No. Cells 100,000/ml	No. Cells 800,000/ml
Complete	0.14	0.17	0.20
-N	.01	.02	.04
" + 0.25	.02	.04	.06
" + 0.5	.04	.06	.08
" + 1.0	.08	.09	.10
-P	.01	.07	.10
" + 0.025	.05	.10	.12
" + 0.05	.08	.11	.12
" + 0.10	.11	.14	.14
-Mg	.03	.04	.10
" + 0.025	.08	.08	.13
" + 0.05	.10	.12	.16
" + 0.10	.14	.16	.20
-S	.02	.02	.13
" + 0.025	.04	.04	.13
" + 0.05	.05	.06	.15
" + 0.1	.08	.09	.15
-Fe	.03	.06	.12
" + 0.001	.05	.06	.11
" + 0.002	.04	.06	.15
" + 0.005	.05	.07	.12

responding to the addition of essential nutrients to media lacking that nutrient. By using lower initial cell densities and relatively sensitive biomass measurements, responses to relatively lower concentrations of nutrients could be detected.

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AEROBIC LAKE MUDS FOR THE REMOVAL OF PHOSPHORUS FROM LAKE WATERS

ABSTRACT

Phosphorus-limited Selenastrum and Cladophora sp. have been shown to respond by growth or extractable PO_4-P to as little as 0.02 mg PO_4-P. However, these same species did not respond when exposed for a period of 1 or 2 weeks to as much as 2 mg of phosphorus in lake muds under aerobic conditions. Studies of the rate of sorption of phosphorus by lake muds under aerobic conditions from two lakes and three depths in one lake indicated that as little as 0.4 g of mud could sorb about 0.05 mg PO_4-P in less than 30 minutes. Due to these findings, it is suggested that the sorption of phosphorus by lake muds under aerobic conditions can be used to remove phosphorus from lake water and to demonstrate the importance of the control of continuous sources of phosphorus to lakes.

INTRODUCTION

Bioasssays with Cladophora sp. and other algae have indicated these algae did become phosphorus-limited or nitrogen-limited in Lake Mendota, Monona Bay, and Lake Wingra of Madison, Wisconsin during the summers of 1968 (Fitzgerald and Lee, 1970) and 1969 despite the fact the algae were located close to muds which contained both phosphorus and nitrogen compounds. Therefore, the availability of phosphorus from lake muds for the nutrition of algae was of interest. Mortimer (1941) produced evidence that phosphorus is adsorbed strongly on ferric hydroxide or ferric hydroxide organic complexes in the oxidized surface mud layers of lake bottoms as long as oxygen is present in the overlying water. Harter (1968) and others have studied the adsorption of phosphorus by lake sediments, and Lee (1969) has recently reviewed this subject. Without trying to study the various forms of phosphorus that may be present in lake muds, tests have been carried out to determine if the phosphorus of lake muds is readily available to algae under aerobic conditions. When preliminary tests indicated that the phosphorus was not available to algae in short periods of time, further studies on the use of lake muds to remove phosphorus from solutions were carried out with the objective of evaluating this as a means of removing nutrients from lake waters.

METHODS AND MATERIALS

The PAAP (Bartsch, 1969) strain of Selenastrum capricornutum was cultured in modified Gorham's medium (Hughes et al., 1958) which contained 0.2 mg P/L so the resulting culture was phosphorus(P)-limited. Growth of the algae was measured by absorbance (1 cm, 750 mμ). Absorbance of 0.1 is equivalent to 2-6 million cells/ml, depending on their size, and about 50 mg/L dry weight. Cladophora sp. from Lake Wingra, Madison, Wisconsin, had been collected and tested for surplus-P or P-limited conditions by the one-hour boiling-water extraction method of Fitzgerald and Nelson (1966). The samples used were P-limited because only 0.03 mg PO_4-P were extracted from 100 mg algae.

Mud samples were collected at mud-water surfaces from Madison, Wisconsin lakes by hand or by use of an Ekman dredge. Lake Wingra and Lake Mendota (3-meter depth) samples were collected from weed beds. The sample from the 18-meter depth of Lake Mendota was from the hypolimnion and was anaerobic when collected. Dried mud samples were heated at 110°C for 1 hour or more and ashed samples were heated at 600°C for 1 hour. The approximate dry weights of 25 ml mud samples used in most experiments are presented in Table 3.

The PO_4-P analyses were by the molybdate-stannous chloride method (APHA, 1965), and the approximate total P analyses were by the acid-persulfate method of Gales et al. (1968).

RESULTS

Availability of Phosphorus in Lake Muds to Algae

In order to determine if Selenastrum could use the phosphorus (P) of lake muds for immediate growth, P-limited Selenastrum cells were added to 150 ml of Gorham's (-P) medium (100,000 cells/ml) in aerated tubes (pH maintained at 7.5-8.1 by addition of 0.5% CO_2 in air). Either PO_4-P or lake muds as sources of P were placed in dialysis tubes in the cultures, and the growth of algae after 7 and 12 days was measured by absorbance. Controls with zero or 0.030 mg PO_4-P were used to test the response of the inoculum to the test conditions. Five experiments were carried out. The results of a typical experiment are presented in Table 1 which shows the averages of growths attained in duplicate cultures.

Table 1. Lake Muds as Sources of Available Phosphorus for Selenastrum capricornutum (PAAP). Basal Medium, Gorham's (-P) Medium; 150 ml/Tube; pH Maintained at 7.5-8.1 by Continuous Aeration with 0.5% CO_2 in Air; Initial Cell Density, 100,000 Cells/ml; Fresh Mud Samples in Dialysis Tubes in Cultures.

Added Phosphorus Source	Original Total P (mg P/Tube)	Ave. Growth of Algae as Absorbance (1 cm, 750 mμ)	
		7 Days	12 Days
None	0.00	.005	.005
PO_4-P	0.03	.17	.18
L. Wingra Mud (1/2 m) A	0.09	.005	-
L. Wingra Mud (1/2 m) A + 0.03 mg PO_4-P on Day 7	-	-	.040
L. Wingra Mud (1/2 m) B	0.16	.005	.005
L. Mendota Mud (5 m) A	0.22	.005	-
L. Mendota Mud (5 m) A + 0.03 mg PO_4-P on Day 7	-	-	.090
L. Mendota Mud (5 m) B	0.44	.005	.005
L. Mendota Mud (18 m) A	0.68	.025	-
L. Mendota Mud (18 m) A + 0.03 mg PO_4-P on Day 7	-	-	.070
L. Mendota Mud (18 m) B	1.4	.040	.030

The results indicate that little or no growth was supported by the muds, whereas the algae exposed to 0.03 mg PO_4-P nearly reached their peak yield in 7 days. Since negative results with the mud samples might indicate toxicity under these conditions, PO_4-P (0.03 mg) was added to 3 of the cultures containing muds which had been incubated for 7 days. The results after an additional incubation period of 5 days indicated that the algae-mud cultures which had been given additional PO_4-P were able to grow, so this ruled out toxicity as the reason for lack of growth in the presence of the muds. The mud sample from the 18-meter depth in Lake Mendota provided some P for Selenastrum. The amount of growth attained in these samples (absorbances of 0.025 and 0.04) is equivalent to the growth to be expected from less than 0.01 mg PO_4-P. Since the two mud samples contained 0.68 and 1.4 mg total P, less than 1% of the total P of these samples was available to the algae tested under these conditions. Similar tests also indicated that lake muds did not provide available nitrogen to Selenastrum and other algal species under equivalent conditions.

Since tests with Selenastrum might be prejudiced by the use of laboratory algae and by using dialysis tubes for the mud so that intimate contact with the muds was not possible, additional tests were carried out using P-limited Cladophora sp. from Lake Wingra. With the use of 250 ml Erlenmeyer flasks as containers, the P-source being tested and approximately 10 mg of Cladophora were added to 150 ml Gorham's (-P) medium. In all mud cultures, the Cladophora were partially embedded in the mud. After an incubation period of 2 days in the culture room, the Cladophora were removed, washed, and extracted by the boiling water method. The average amounts of PO_4-P and total P extracted from triplicate cultures are presented in Table 2.

The data indicate that the Cladophora were initially P-limited but that they were able to obtain measurable amounts of available P from even the lowest level of PO_4-P added (0.02 mg P). The amount of PO_4-P extracted from algae of cultures with increasing PO_4-P levels increased proportionately to the added P up to 0.04 mg P. A level of 0.08 mg P appears to have been surplus for the amount of algae present (10 mg). The lack of any increase of extractable P in the algae in the presence of the mud from Lake Wingra and the 3-meter depth of Lake Mendota indicates that none of the 0.8 and 1.2 mg of total P present was available to the Cladophora. The mud sample from the 18-meter depth of Lake Mendota provided P to the Cladophora equivalent to 0.02 mg PO_4-P. Therefore, only 1% of the total P of this sample was available.

Table 2. Lake Muds as Sources of Available Phosphorus for Cladophora sp. Basal Medium, Gorham's (-P) Medium; 150 ml/250 ml Erlenmeyer Flasks; Approx. 10 mg P-Limited Cladophora (0.03 mg PO_4-P Extracted/ 100 mg Algae) from L. Wingra; Cladophora Partially Embedded in Mud Samples at Bottom of Flask.

Phosphorus Source	Phosphorus Added (mg Total P/ Culture)	Ave. Phosphorus Extracted from Cladophora after 2 Days (mg P/10 mg Algae)	
		PO_4-P	Total P
None	0.00	0.0032	0.006
PO_4-P	0.02	0.0086	0.018
PO_4-P	0.04	0.018	0.024
PO_4-P	0.08	0.021	0.028
L.Wingra Mud (1/2 m)	0.8	0.0033	0.008
L.Mendota Mud (3 m)	1.2	0.0030	0.009
L.Mendota Mud (18 m)	2.2	0.0090	0.018

The Sorption of Phosphorus by Lake Muds

Since the above tests and bioassays with Cladophora sp. and other algae from the Madison lakes indicated that lake muds under aerobic conditions did not provide algae with adequate available phosphorus, tests of the sorption of PO_4-P by lake muds were carried out. In the first series of experiments different amounts of lake muds were added to 150 ml of Gorham's (-P) medium in aerated tubes and the amounts of PO_4-P in the supernatants were analyzed at different times. Initial tests indicated that less than 15 µg PO_4-P/150 ml were released during a period of several days. The amounts of PO_4-P remaining in the supernatants after the addition of 300 µg PO_4-P were then tested. The results from a typical experiment are presented in Table 3 which shows average percentages of P sorbed by the lake muds.

It is evident that lake muds from aerobic areas of the lakes as well as muds from anaerobic depths would sorb PO_4-P from solutions under these aerobic conditions. A good proportion of the PO_4-P sorption took place in the first 10 minutes under these conditions, but when similar tests were carried out with the muds encased in dialysis tubes, at least 24 hours were required for 50% or more sorption. Thus, it becomes apparent why PO_4-P added to Selenastrum cultures is not competitively sorbed by lake muds in dialysis tubes in the cultures since the sorption reaction through dialysis tubing is so slow and Selenastrum can effectively absorb maximal quantities of phosphorus within 1 or 2 hours of exposure (Fitzgerald, 1970).

As various studies have mentioned different sorption reactions, the sorption of P by lake muds that had been dried or ashed was compared with the sorption by untreated samples. The averages of the percentage of P added to 150 ml of Gorham's (-P) medium which was sorbed by treated mud from the 18-meter depth of Lake Mendota are presented in Table 4.

The amount of PO_4-P added and the treatments given the mud from Lake Mendota appear to have an effect on the sorption of P. The percentage of P sorbed by 2.8 g of mud decreased as the amount of PO_4-P added increased, but even 60% of 1,200 µg of PO_4-P could be sorbed from solution by fresh samples within 10 minutes. The effect of drying on the sorption reaction was to cut the rate in half. However, the rate of sorption by ashed samples was very high. With samples from Lake Wingra and other depths of Lake Mendota, ashed muds were also found to sorb significant amounts of P in less than 2 minutes. Therefore, the sorption of P by

Table 3. Rate of Sorption of Phosphorus by Different Aerobic Lake Muds. Gorham's (-P) Medium; 150 ml/Aerated Tube; pH 7-8; Supernatants of Mud-Containing Tubes Without Added P Contained Less Than 15 µg PO_4-P/150 ml at Any Time; 300 µg PO_4-P Added to 150 ml Medium Plus Mud and PO_4-P Remaining in Supernatant Analyzed after Different Times.

Mud Source	Mud Added (g[Dry Weight]/ 150 ml)	Ave. Percentage Phosphorus Sorbed		
		10 min	60 min	180 min
L. Wingra (1/2 m)	17	30	65	80
L. Mendota (3 m)	22	40	65	75
L. Mendota (5 m)	13	20	25	30
L. Mendota (18 m)	3	70	80	85

Table 4. Rate of Sorption of Phosphorus by Treated Bottom Mud (18 meters) of Lake Mendota under Aerobic Conditions. Gorham's (-P) Medium; 150 ml/Aerated Tubes; pH 7-8; Supernatants of Tubes of Mud without Added P Contained Less Than 30 µg PO_4-P at Any Time; 300 µg or More PO_4-P Added and PO_4-P Remaining in Supernatant Analyzed after Different Times.

Mud Treatment	Mud Added Dry Weight (g/150 ml)	Phosphorus Added (µg/150 ml)	Ave. Percentage Phosphorus Sorbed	
			10 min	60 min
Fresh	2.8	300	75	90
Fresh	2.8	600	70	80
Fresh	2.8	1200	60	70
Dried	2.8	300	35	50
Ashed	2.3	300	95	95

lake muds is not related to the organic material present (0.5 g of volatile solids per 2.8 g dry solids).

The tests reported thus far were carried out with relatively large amounts of lake muds added to 150 ml of Gorham's (-P) medium. Further tests with lesser quantities of muds and in other media were also carried out. Gorham's (-P) medium contains over 700 mg dissolved solids/liter, but PAAP (-P) medium has less than 200 mg/liter. Therefore, natural waters and the more dilute PAAP (-P) medium were used. Different amounts of mud from the 18-meter depth of Lake Mendota were added to the test media which contained 1 mg PO_4-P/liter and the PO_4-P removed from the solution analyzed. The results of a typical experiment with PAAP (-P) medium showing the average percentage of PO_4-P sorbed after 30 and 60 minutes are presented in Table 5.

The data indicate that the amount of PO_4-P sorbed from any volume increases as the amount of mud added increases. With an increase in the volume of the water in which a quantity of mud is suspended, the percentage of PO_4-P sorbed decreases. Similar results were also obtained when different lake waters were used. One of the lake waters tested had 90 µg PO_4-P/liter present, and this amount was reduced to less than 20 µg/liter by the muds within 30 minutes, so the PO_4-P of lake water appears to be sorbed in the same manner as added PO_4-P. Therefore, these quantitative results would be of value in predicting the amount of mud which would have to be used to sorb particular quantities of P from lake waters.

DISCUSSION

The early onset of phosphorus-limitation of the Cladophora sp. along the southern shore of Lake Mendota (mid-June in 1968 [Fitzgerald and Lee, 1970] and early June in 1969) has indicated that the phosphorus supplies coming from winter degradation, spring runoff, and lake overturn do not remain available in the surface waters of a lake for the nutrition of algae restricted to these waters. The manner in which available phosphorus is lost from solution is, therefore, of considerable interest in studies of eutrophication. PO_4-P has been shown to be lost from sewage plant effluents in oxidation ponds during relatively high pH conditions associated with the growth of algae, but the solubilization of the phosphorus when pH values decreased towards pH 8 indicated that the original loss from solution must have been by precipitation (Fitzgerald, 1961). The loss of phosphorus added to fish ponds reported by Hepher (1965)

Table 5. Rate of Sorption of Phosphorus by Bottom (18 meter) Mud from Lake Mendota under Aerobic Conditions. PAAP Medium (-P); pH 7-8; Aerobic Mud Samples Added to 1 mg P/L.

Sample Volume (ml)	Mud Added (grams[dry wt])	PO_4-P Added (µg)	Ave. Percentage Phosphorus Sorbed	
			30 min	60 min
50	0.1	50	30	50
50	0.2	50	70	70
50	0.4	50	80	90
100	0.2	100	30	50
100	0.4	100	60	70
100	0.8	100	80	90
250	0.2	250	20	30
250	0.4	250	30	40
250	0.8	250	50	60

also seemed to be due to precipitation as tricalcium phosphate. However, studies on the availability of phosphorus which had been precipitated by the addition of relatively high concentrations of calcium and iron or only iron to algal culture media indicated that such precipitated phosphorus was nearly as readily available for the growth of several species of phosphorus-limited algae as soluble PO_4-P (Fitzgerald, 1970). As a matter of fact, when teeth were used as a source of "insoluble" phosphorus at the suggestion of Lee (G. Fred Lee, 1969, personal communication) it was found that dog and shark teeth and even petrified shark teeth supported the growth of several species of phosphorus-limited algae at about the same rate as soluble PO_4-P (Fitzgerald, 1970). Therefore, the adsorption reactions suggested by Mortimer (1941) and others must be of more importance in the loss in availability of phosphorus in lake waters than mere insolubility since the data presented here indicate that the phosphorus of lake muds is not immediately available to phosphorus-limited algae under aerobic conditions.

Since phosphorus is so readily lost from lake waters early in the spring, the importance of winter degradation, spring runoff, and spring turnover as sources of phosphorus to algal ecology is questionable. Therefore, the data presented here and by others (Harter, 1968) showing that lake muds sorb phosphorus under aerobic conditions could be of immense ecological importance. If muds could be used to sorb the phosphorus of surface waters or the epilimnion after stratification takes place but before anaerobic conditions develop in the hypolimnion, the muds could effectively strip the epilimnion of phosphorus and return it to the bottom or to the hypolimnion. There would be less phosphorus in surface waters, therefore, to support obnoxious algal growths. It should be pointed out that studies by Lee (G. F. Lee, 1970, personal communication) have shown that very significant amounts of phosphorus are released from aerobic lake muds when tests are carried out for periods exceeding one month and this release is more rapid under anaerobic conditions. Thus, information on the relative importance of continuous sources of phosphorus to surface waters, such as contamination from waste waters, the regeneration from bottom muds, or decay of plants, is of prime importance.

It should be emphasized that if the available phosphorus of surface waters were to be stripped by the use of bottom muds, the growth of algae might be limited, but such actions would probably have no effect on the growth of rooted aquatic weeds. Lake muds have been shown by Martin *et al.* (1969)

to be main sources of nutrients for the aquatic weed, Najas sp. In the absence of competition with algae (Hasler and Jones, 1949; Fitzgerald, 1969) the growth of aquatic weeds would probably be stimulated beyond normally expected amounts. Therefore, the physical manicuring of weed beds in much the same manner as used to control the growth of grass in parks would be necessary to allow the most benefit to be gained from aquatic environments.

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EVALUATIONS OF THE AVAILABILITY OF SOURCES OF NITROGEN AND PHOSPHORUS FOR ALGAE

ABSTRACT

Techniques are compared for the evaluation of nutrient sources in which the potential nutrients are in contact with algae over their entire culture period versus relatively short-term exposure tests. The availability of relatively insoluble nutrients: iron-phosphorus compounds and teeth for phosphorus, hair for nitrogen, iron pyrites for iron, and marble for carbon, indicates that in many cases the equilibrium between soluble and insoluble forms allows algae to successfully compete with insoluble forms for nutrients. Apparently, factors other than mere insolubility prevent algae from obtaining available nitrogen or phosphorus from phosphate rock or lake muds. The facts, (1) that live algae and aquatic weeds do not share their adequate or surplus nutrients with nutrient-limited algae and (2) that lake muds do not provide readily available nitrogen or phosphorus, indicate that once lake waters are stripped of available nutrients by plant production, further plant production will depend upon nutrients from continuous sources of nutrients, such as wastewater effluents.

INTRODUCTION

In order to determine if a potential source of an algal nutrient is actually available for the growth of algae one usually adds different concentrations of the source to nutrient-limited cultures of algae and compares the growth attained with cultures lacking the nutrient and with the growth of cultures having standard amounts of known available sources of the nutrient. One of the disadvantages of this procedure is that algae grow so slowly that the potential source of nutrient must be present in the medium for a week or more before one can judge whether it was an available source of nutrient under those conditions. During this time of incubation one cannot be sure that the original source has not been degraded to a more available source of nutrient by the conditions of the test, such as the degradation of condensed phosphates to orthophosphate either in the presence or absence of bacteria in lake waters or culture media.[3] Therefore, when possible, it would be desirable to use nutrient-availability tests which would rely on relatively short exposure times to a potential source of nutrient under conditions which can be readily controlled,

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such as pH, light, or temperature, and which represent some of the factors that might be involved under field exposures to the sources of the nutrients.

Relatively short-term exposure periods to potential sources of nutrients under controlled environmental conditions can be followed by removing aliquots of algae after different exposure times and either (1) subculturing them to measure the amount of growth attained after further incubation or (2) measuring an enzymatic change related to the absorption of a nutrient. The type of test to be used will be limited by known reactions with established nutrients. We have demonstrated several different techniques for the evaluation of the availability of nitrogen from sources and a short-term test, using the subculture technique, for evaluating sources of phosphorus.

Besides the physical factors, such as pH, light, and the chemical composition of a potential nutrient source, the solubility of a compound is frequently considered to be of importance in nutrition. In order to evaluate some nutrient sources that potentially might be affected by solubility, we have demonstrated with relatively insoluble nitrogen and phosphorus compounds that in some cases the equilibrium between soluble and insoluble states of some compounds allows adequate nutrients to be available to algae under the usual growth conditions. However, not all potential nutrients, such as the nitrogen and phosphorus of lake muds, are readily available to algae in short-term experiments.

Several workers[12] have demonstrated how certain aquatic animals can provide nitrogen or phosphorus for use by algae. However, little work has demonstrated nutrient-sharing between algae or between algae and aquatic plants of higher forms. In contrast, there are many articles dealing with tracer studies of nutrient exchange. Therefore, studies were made of the availability of the nitrogen and phosphorus of one alga or aquatic weed to another. In other words, conditions under which plants share their nutrients were studied.

These studies are presented in order to demonstrate techniques available for such investigations and to provide information on some of the factors affecting the availability of certain potential nutrient sources. It is hoped that further evaluations will be stimulated from the information presented here.

MATERIALS AND METHODS

The green alga, Selenastrum capricornutum (PAAP; 2), was used as the main test organism. This alga was cultured in modifications either of PAAP medium[2] or of Gorham's medium.[11] The techniques used to determine whether algae were phosphorus-limited[9] or nitrogen-limited[5] have been described previously. The measurements of growth of cultures were usually done by light absorbance measurements (1 cm, 750 mμ). An absorbance of 0.1 is equivalent to 2 to 6 million cells per ml, depending upon cell size, and to about 50 mg/L dry weight. The field collections of algae, aquatic weeds, and lake muds came from Lakes Mendota or Wingra of the Madison, Wisconsin area.

Analyses for NH_3-N were by direct Nesslerization,[1] for PO_4-P by the molybdate-stannous chloride method,[1] and for approximate total phosphorus by the acid-persulfate method of Gales et al.[10] Analyses of the concentration of soluble PO_4-P from various "insoluble" phosphorus sources were carried out at pH 7.5 after filtration through glass-pad filters (Reeve Angel, #934 AH). The percentage of ortho PO_4-P in the commercial phosphorus sources used was analyzed: sodium tripolyphosphate (1.4% ortho PO_4-P), sodium pyrophosphate (2.5% ortho PO_4-PO, and sodium phosphite (0.36% ortho PO_4-P).

RESULTS

Techniques for Evaluation of Nutrient Sources

A common procedure for evaluating a nutrient source is to add nutrient-limited algae to different concentrations of the source, using suitable standards as guides. The growth attained after incubation is then measured. This method was used to evaluate different sources of nitrogen for Selenastrum. Selenastrum from PAAP medium were placed in PAAP (-N) medium for 2 additional days to ensure that cells were N-starved. These algae then were added to 150 ml of medium in aerated (air + 0.5% CO_2) tubes at pH 7 to give an initial concentration of 100,000 cells/ml. The growths as absorbances after different incubation times with ammonia, nitrate, nitrite and glutamic acid in a typical experiment are presented in Table 1.

The data indicate that the rate of utilization of ammonia was slightly less than for the other sources (i.e., data for 5 and 6 days), but the pH of the cultures with 10 mg NH_3-N/L was lower (pH 6.3 versus 6.8) after incubation for

Table 1. Utilization of Nitrogen Sources by Selenastrum. PAAP (-N) Medium, 150 ml/Tube, pH 7.

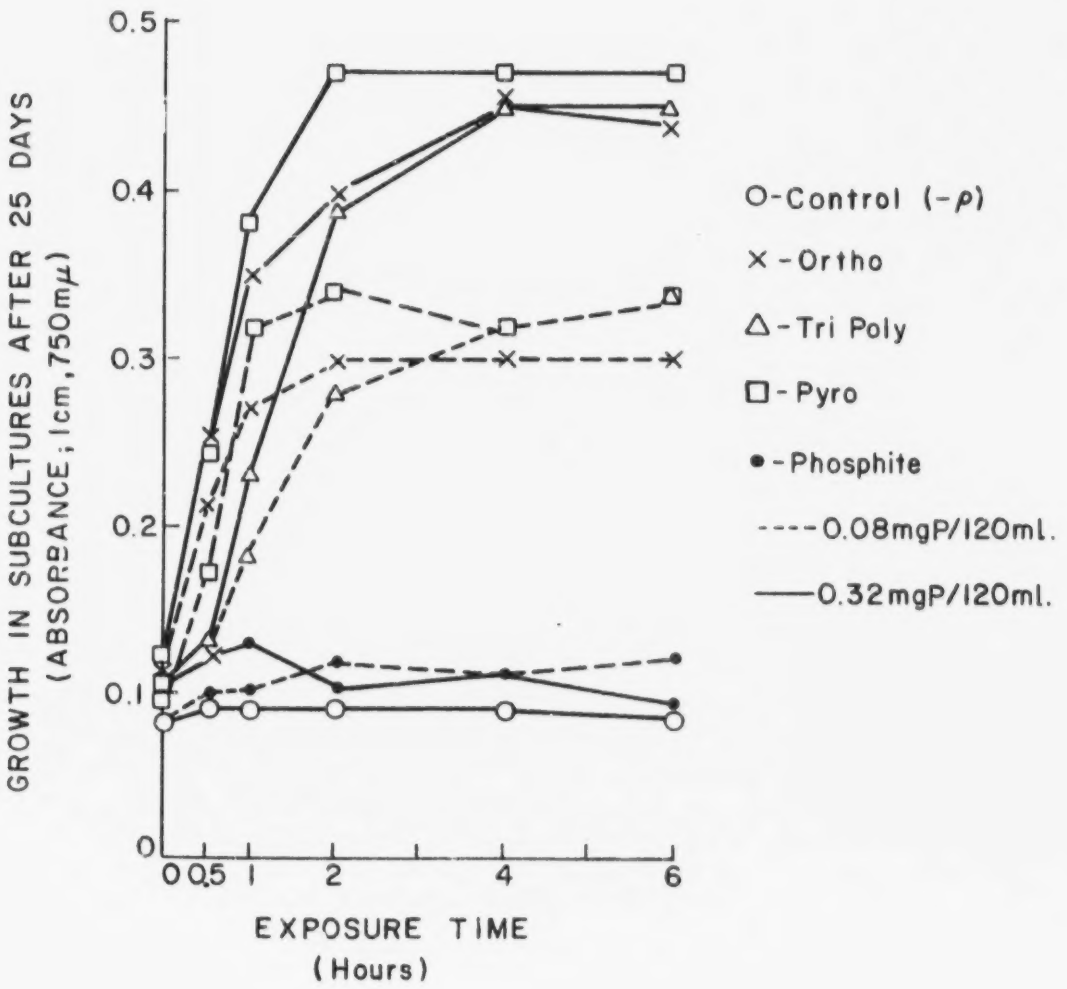
N Source	mg N/L	Growth of Algae as Absorbance of Cultures (1 cm, 750 mμ)		
		5 Days	6 Days	11 Days
None	0	0.015	0.015	0.015
Ammonia	5	.06	.13	.28
	10	.06	.15	.53
Nitrate	5	.11	.22	.30
	10	.15	.36	.55
Nitrite	5	.13	.26	.36
	10	.14	.36	.58
Glutamic Acid	5	.06	.12	.19
	10	.15	.26	.38

6 days. After 11 days of culture the growths in ammonia, nitrate and nitrite were essentially the same at each nitrogen level, but the growth with glutamic acid-nitrogen was about 65% that of the other nitrogen sources. Because the degradation or conversion of the nitrogen sources before utilization could not be ruled out, and because pH was not consistent, these results were not considered to be very reliable.

The physical conditions of tests can be more readily controlled when short-term exposures of nutrient-limited algae to potential nutrient sources are used. In order to demonstrate this technique, phosphorus-limited or nitrogen-limited algae were exposed to different nutrients under controlled conditions and the subsequent growth of subcultures was used to evaluate the relative availability of the nutrients. Phosphorus sources were evaluated by using Selenastrum grown in PAAP medium plus at least 2 days in PAAP(-P) medium so they were phosphorus-limited at the time of use. The algae were placed in aerated tubes with different levels of phosphorus (0, 0.08, and 0.32 mg P/150 ml), and after different exposure times, duplicate 0.5 mg (dry weight) samples were removed, washed and placed in 25 ml of sterile PAAP(-P) medium for incubation. Growth attained in the subcultures for a typical experiment evaluating ortho PO_4-P, tripolyphosphate, pyrophosphate, and phosphite are presented in Figure 1 as the averages of duplicate absorbances attained after 25 days.

It was found that Selenastrum would not absorb and utilize phosphite-phosphorus in either light or dark exposures of up to 26 hours; little or no growth occurred in subcultures, and there was no loss in phosphite concentration in the exposure tubes. This contrasted to no measurable phosphorus remaining in the supernatants of the other phosphorus sources. Ortho PO_4-P and pyrophosphate appeared to be equally effective as sources of phosphorus. The apparent increased availability of pyro is felt to be insignificant. The rate of utilization of tripolyphosphate was less than that of PO_4-P and pyrophosphate; the growth attained in subcultures after 1/2- to 1-hour exposures was about 50% that of PO_4-P and pyrophosphate subcultures. Growth of subcultures was essentially the same after 4- to 26-hour exposures. The results of tests in which the exposure tubes were held in darkness during the sampling periods were similar to the results obtained when tubes were held in the light (600 ft.C). Exposure tests in the light at pH 7 (6.8-7.3) and pH 9 (8.8-9.3) gave equivalent results: PO_4-P was absorbed more rapidly than tripolyphosphate, but with

Figure 1. The Effectiveness of Different Phosphorus Sources for the Growth of Selenastrum by the Exposure and Subculture Technique. Averages of Duplicate Cultures. PAAP (-P) Medium, pH 7-7.5, 400 ft. Candles.



longer exposures the tripolyphosphate was as good a phosphorus source as PO_4-P.

When the exposure and subculture technique was used with nitrogen-limited Selenastrum, it was found (Table 2) that the subsequent growth of 0.5 mg samples from algae exposed to ammonia, nitrate, nitrite and urea (3.2 mg N/120 ml) in 600 ft.C light resulted in light absorbances after incubation for 3 weeks of 0.06 for control (-N) samples and 0.16 to 0.22 for exposures of 24 hours to the nitrogen sources. The rates of utilization, as measured by the amount of growth attained by the 0.5 mg samples taken at increasing exposure times, were so close to each other for the different nitrogen sources that this method could not detect any differences in availability of these nitrogen sources.

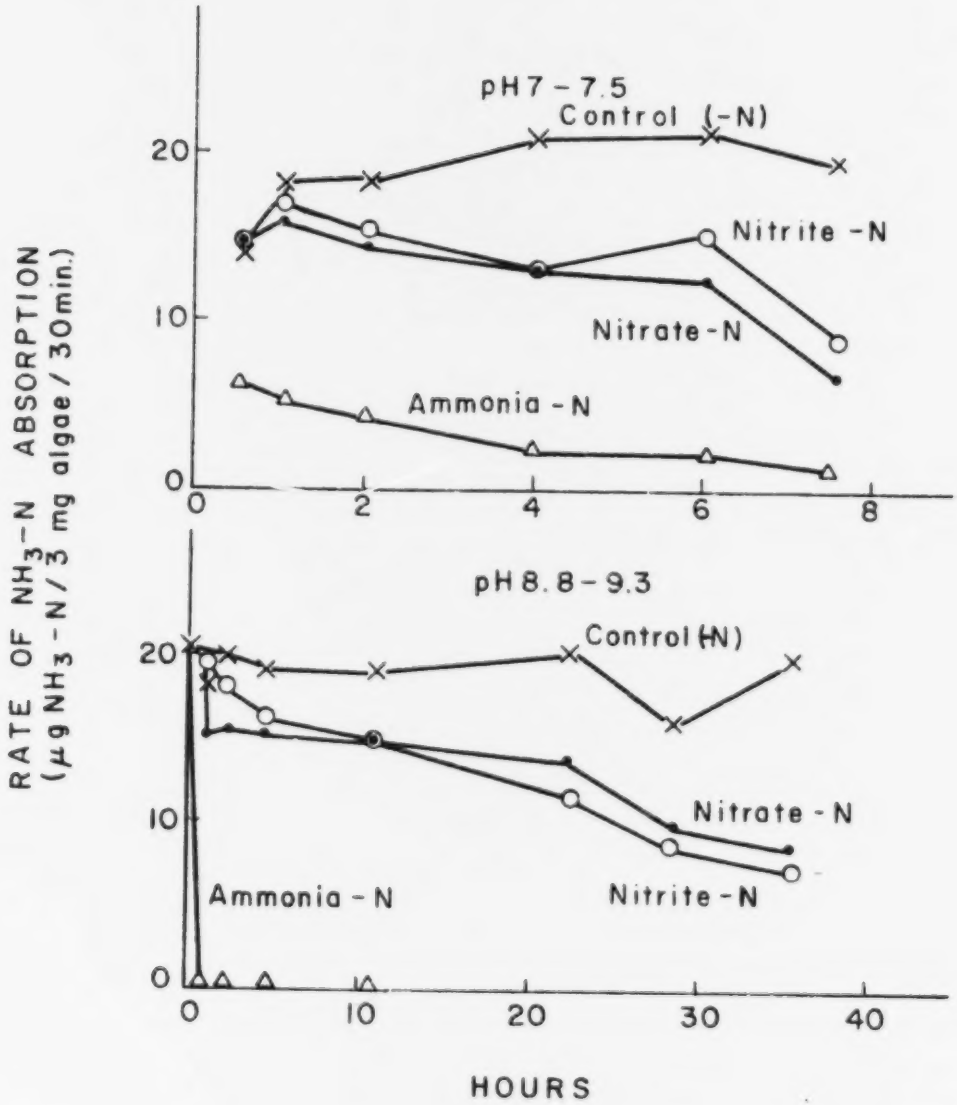
The distinct disadvantage of the techniques demonstrated thus far has been that results are not apparent until after a 2- to 3-week incubation period of the algae. Results could be determined more rapidly if changes produced in the algae were followed. Such tests could not replace the ultimate test of the growth of algae with absorbed nutrients, but could be used for rapid evaluations under specific environmental conditions. One such test is to follow the absorption of phosphorus by measuring changes in the amount of extractable phosphorus from algae after exposure to different concentrations of phosphorus sources, as described in a later section. Another short-term technique is to measure the effects of sources of nitrogen on the rate of NH_3-N absorption in the dark by nitrogen-limited algae. In a typical series of tests, nitrogen-limited Selenastrum were added to test media (24 mg algae/80 ml Gorham's (-N) medium so that 3 mg samples could be removed after different exposure times and then washed, and the amount of NH_3-N absorbed in the dark over 30 minutes measured. A summary of typical results of at least 3 tests with 0 and 7.5 mg ammonia-, nitrate-, or nitrite-nitrogen in the light (600 ft.C) at pH 7 versus pH 9 is presented in Figure 2.

In all tests the algae from control exposure tubes lacking any fixed nitrogen (-N) had NH_3-N absorption rates of 15-20 µg NH_3-N/3 mg algae/30 minutes which indicated they were nitrogen-limited.[5] The effect of added ammonia was detected within 30 minutes, but detection of added nitrate- and nitrite-nitrogen required longer exposure times. Ammonia was much more effective as a nitrogen source by this technique at pH 9 than at pH 7, whereas nitrate- and nitrite-nitrogen required less time to affect the NH_3-N absorption rate at pH 7 than at pH 9 with Selenastrum. Similar results were obtained if the exposures to the nitrogen sources were

Table 2. Utilization of Nitrogen Sources by Selenastrum as Measured by Growth Attained by 1/2 mg Samples Placed in 25 ml PAAP (-N) Medium after Different Exposure Times. 400 ft. C., pH 7, 3 Weeks Incubation. Exposure: 3.2 mg N/16 mg Algae/120 ml PAAP (-N).

N Source	Effect of Exposure Time on the Average Growth Attained (Absorbance, 1 cm, 750 mμ)		
	1 Hour	6 Hours	24 Hours
None	0.06	0.06	0.06
Ammonia	.08	.10	.21
Nitrate	.08	.08	.16
Nitrite	.09	.11	.16
Urea	.08	.11	.22

Figure 2. The Effect of Different Nitrogen Sources on the Ammonia Absorption Rate of Selenastrum. Averages of Duplicate Cultures. Gorham's (-N) Medium, pH 7-7.5 versus pH 8.8-9.3, 600 ft Candles.



carried out in light or in darkness. This technique would appear to be suitable, therefore, for comparative tests with potential nitrogen sources and with relatively short exposure times necessary.

Solubility as a Nutritional Factor

Solubility should be added to the physical factors, pH, light, and chemical structure, which might affect the availability of a nutrient. Solubility is not a simple factor related only to whether algae can compete with the equilibrium between a soluble and insoluble form of a nutrient, but consideration of it is complicated by the fact that if a nutrient is insoluble it probably will tend to settle out of suspension and away from the algae. Therefore, only speculation can be made of the effect of solubility in a lake environment until more knowledge is gained as to the location and manner of absorption of nutrients by algae. However, tests can be made of the availability of soluble versus insoluble nutrients in algal cultures, and a few such examples are presented.

The solubility of phosphorus in PAAP medium (minus EDTA) can be manipulated by the addition of $FeCl_3$: 0.2 mg P/L is 100% soluble in PAAP; 50% soluble in PAAP + 2.5 mg Fe/L; and 15% soluble in PAAP + 5 mg Fe/L. The relative rate of growth of Selenastrum in 0.06 to 0.4 mg P/L was measured when the phosphorus was soluble or partially insoluble and when the phosphorus was in the algal growth medium or separated from the algae by semipermeable membranes.

In the simplest experiment the rate of growth of Selenastrum (100,000 cells/ml initially) was followed in the three media mentioned above, such that the phosphorus was soluble, 50% soluble, and 15% soluble. These tests were carried out in 150 ml of medium in tubes aerated with 0.5% CO_2 in air in order to maintain pH 7-7.5. The growth of Selenastrum in levels of 0.06 to 0.4 mg P/L is presented as light absorbances in Table 3.

The data indicate that despite a range of solubility of the available phosphorus from 100% to 15%, the rate of growth was the same between 4 and 8 days at all levels of phosphorus, and the maximum growth attained was independent of the media at phosphorus levels of 0.27 and 0.4 mg/L.

Since the above results might be prejudiced by the fact that the algae were in intimate contact with the insoluble form of phosphorus so that one could not separate the effect of

Table 3. The Effect of Solubility of Phosphorus on the Growth of Selenastrum. (150 ml, pH 7-7.5)

Medium	% P Soluble[1]	mg P/L	Ave. Growth as Absorbances (1 cm, 750 mμ)			
			4 Days	6 Days	7 Days	8 Days
PAAP	100	0.067	0.07	0.10	0.11	0.11
		0.13	.09	.16	.18	.19
		0.27	.08	.19	.22	.24
		0.40	.10	.23	.27	.31
PAAP + 2.5 mg Fe/L	50	0.067	.02	.03	.04	.05
		0.13	.06	.13	.14	.15
		0.27	.06	.25	.32	.33
		0.40	.07	.31	.40	.44
PAAP + 5 mg Fe/L	15	0.067	.00	.01	.01	.02
		0.13	.01	.02	.05	.08
		0.27	.03	.19	.27	.31
		0.40	.04	.33	.41	.45

[1]Solubility at 0.2 mg P/L Level

algal surface enzymes on the insoluble phosphorus from the effects of competition of the algae with the equilibrium between soluble and insoluble forms of phosphorus, a series of experiments were carried out in which the phosphorus was placed in dialysis tubing or on the opposite side of a membrane filter (Millipore, 0.45 μ) from the Selenastrum. When 0.05, 0.10 and 0.20 mg P were placed in about 30 ml of PAAP medium inside dialysis tubing and the tubing placed in 750 ml of PAAP medium in one-liter Erlenmeyer flasks, the growth of Selenastrum (initially 100,000 cells/ml) in the culture flask attained absorbances of 0.13, 0.15 and 0.27 after 5 days. When the basal medium in the tube and culture was PAAP + 5 mg Fe/L (solubility of 0.2 mg P/L = 15%), the same levels of phosphorus resulted in absorbances of 0.03, 0.15, and 0.32 after 5 days. Similarly, the rate of growth of Selenastrum in the medium in which the phosphorus was only 15% soluble was approximately the same as in the PAAP medium when the phosphorus was placed on the opposite side of a membrane filter from the algae (500 ml Bellco spinner flask #3008, 0.45 μ Millipore filter).

We infer from these data that the solubility of phosphorus in culture media in which the reaction of iron and phosphorus may result in different solubilities of the phosphorus is not a limiting factor for the growth of algae. The chemical equilibrium between insoluble and soluble phosphorus under these conditions is apparently such that phosphorus is available for algal growth; the rate of solubility of phosphorus from the iron-phosphorus complex is faster than the rate of growth of the alga, Selenastrum. Similar tests using phosphorus which had been precipitated with relatively high levels of both calcium and iron at pH 9-9.5 also indicated that insoluble phosphorus was readily available for the growth of Chlorella pyrenoidosa (Wis. 2005).

Lee (G. Fred Lee, 1969, personal communication) suggested that forms of insoluble nutrients from nature be tested for availability of nutrients. Suggestions included: teeth and rocks for phosphorus, hair for nitrogen, and iron pyrites for iron. Accordingly, we have tested various types of teeth as sources of phosphorus for Selenastrum. A dog tooth (poodle, puppy, Sherry's Silver Dusay, a 70 mg canine tooth), a shark tooth (unidentified, 2.9 g) and 3 petrified shark teeth (unidentified, 1.7 g total), when placed in PAAP (-P) medium inoculated with 100,000 Selenastrum cells/ml, or separated from the algae by dialysis tubing, supported the rate of growth and ultimate growth attained by at least 0.2 mg ortho PO_4-P/L. Repetition of these tests gave the same results each time, so it was concluded that washing

the teeth or repetitive growth of algae did not exhaust the supply of phosphorus supplied by the teeth.

Hair was used to demonstrate "insoluble" N sources. The results from one test in which various sources of well rinsed hair were added directly to Gorham's (-N) cultures of N-limited Selenastrum (100,000 cells/ml) are presented in Table 4.

It was found that the rate of growth of the Selenastrum appeared to be faster in the control culture with 5 mg NO_3-N/L than with the various hairs, but hair from a cat and two dogs appeared to support good growths. The human hair used in this test had been cut about 2 years before the test, so an additional test with fresh hair was also carried out, but the results with as much as 100 mg (dry weight) indicated human hair to be a relatively poor nitrogen source. Since such negative results might indicate a toxicity reaction, NO_3-N (5 mg/L) was added to a culture containing 20 mg of human hair in which the Selenastrum had not grown appreciatively over a 7-day period. Within 24 hours the absorbance of the culture had increased from 0.01 to 0.05 and the culture had become green.

During the course of experiments on the availability of sources of iron, two series of tests were carried out with natural crystals of iron pyrites which were assumed to be very insoluble because they had resisted solution under natural conditions in the river in which they were found. It was found that although control PAAP (-Fe, +EDTA) cultures attained barely measurable growth, Selenastrum were able to use the iron pyrites as a readily available source of iron, and the resulting growth was equivalent to that supported by the $FeCl_3$ usually used.

Similar tests of carbon sources for Selenastrum and the bloom-forming blue-green alga, Microcystis aeruginosa (Wis. 1036), have indicated that under conditions in which carbon was a limiting nutrient in cultures in PAAP or Gorham's medium (flasks 1/2-1/3 filled with medium and closed with rubber stoppers) the carbon from increasing concentrations of either calcium carbonate or marble, despite their being relatively insoluble sources of carbon, could support increasing amounts of growth of these algae.

As an approach to more ecologically important sources of algal nutrients, samples of Florida phosphate rock (Nat. Bur. Std. #120 a) (0.9% of phosphorus as soluble P in PAAP medium) were added to Selenastrum cultures (100,000 cells/ml,

Table 4. Hair as a Source of Available Nitrogen for *Selenastrum*. Gorham's Medium (-N), pH 7, 150 ml/Tube.

N Source	Dry Weight (mg/Tube)	Growth of *Selenastrum* as Absorbance (1 cm, 750 mμ)	
		5 Days	9 Days
None	0	0.02	0.02
NO_3-N	0.75	.31	.42
Human (Brown)	10	.02	.03
" "	20	.02	.02
" "	40	.04	.03
Cat (Persian)	10	.04	.08
" "	20	.07	.16
" "	40	.15	.36
Dog (Irish Wolfhound)	10	.04	.06
" " "	20	.08	.13
" " "	40	.14	.22
" (Poodle)	20	.06	.10

150 ml PAAP [-P]). This rock was either dispersed in the cultures or separated from the algae by dialysis tubing so as to provide as much as 5 mg of total P/L for the cultures. It was found that only a slight growth of Selenastrum was supported by the rock source of phosphorus; up to 5.0 mg total P/L supported as much growth as 0.05 mg ortho PO_4-P/L. Ultrasonic treatment of the rock suspension did not increase the availability of the phosphorus. The addition of 3 petrified shark teeth to one rock culture which had supported little growth for 1 week resulted in the tripling of the amount of algae after 4 days of further incubation. It was concluded that only a fraction of the total phosphorus of this rock was available for Selenastrum, and this conclusion is in contrast to the results with relatively insoluble sources of phosphorus tested before. In other words, some factor other than simple insolubility was inhibiting the availability of the phosphorus of the Florida phosphate rock.

In experiments[6] evaluating the immediate availability of phosphorus of lake muds under aerobic conditions for the growth of algae in short-term experiments, it was found that whereas phosphorus-limited Selenastrum nearly reached their peak yield in 7 days with 0.03 mg PO_4-P/150 ml cultures, cultures containing as much as 2 mg of total phosphorus in lake muds supported little or no growth when muds from 2 lakes and from 3 depths of one of the lakes were tested. Since negative results might indicate toxic conditions in these experiments, additions of PO_4-P (0.03 mg) were made after 7 days of incubation to 3 of the cultures containing muds. The results of an additional incubation period of 5 days indicated that the algae-mud cultures were able to grow with the additional PO_4-P, so this ruled out toxicity as a reason for lack of growth in the presence of muds. Additional tests with field collections of phosphorus-limited Cladophora sp. also indicated this alga could not obtain increased extractable phosphorus compounds from 48-hour exposures to various lake muds, but could with added PO_4-P. When lake muds were tested for available nitrogen it was repeatedly found that as much as 8 mg of lake-mud nitrogen did not support measurable growth of Selenastrum, but that 0.8 mg NO_3-N supported good growth (absorbance of 0.3).

It is apparent, therefore, that while certain relatively insoluble forms of nutrients may be readily available for the nutrition of algae, certain natural forms of phosphorus or nitrogen, such as phosphate rock and lake muds, were not readily available sources of nutrients.

Factors in the Sharing of Nutrients Between Plants

Observations and results of field tests[8] have suggested that algae do not effectively share their nutrients with one another. It is well known that under certain circumstances the nitrogen from N_2-fixing algae can be found in other plants,[13] and it is generally assumed that algae could be the base of organic matter production in recently denuded areas. However, the question of whether one viable alga or plant will effectively support the growth of another within short-time periods under natural conditions has had only negative answers thus far.[8] Therefore, laboratory tests have been carried out to determine what factors influence the sharing of nutrients of one algal species with another.

A series of tests were carried out in which the potential sources of nitrogen or phosphorus were either placed in the cultures or separated from the cultures by dialysis tubes, and the growth of nitrogen-limited or phosphorus-limited Selenastrum (100,000 cells/ml) under these conditions was compared. Preliminary results indicated that live algae did not share their nutrients. Therefore, more detailed tests were carried out in which live algae and algae gently killed by freeze-thawing were compared as to the availability of their nitrogen or phosphorus. The freeze-thawing technique was shown to kill 99% or more of Selenastrum tested. The amounts of algae added as nutrient sources were calculated as dry weights taken from equivalent amounts of algae. The results of the tests are presented in Tables 5 and 6 as absorbances of cultures and summarized as the calculated average absorbance per 10 mg of added algae.

The data of Table 5 indicate very little growth of nitrogen-limited Selenastrum was supported by live algae, but killed algae which were not nitrogen-limited supported good growths of this alga. Very little growth was attained in cultures which depended upon nitrogen from samples either from the nitrogen-limited Cladophora sp. from Lake Mendota or from the nitrogen-limited Selenastrum from a Gorham's medium containing only 7.5 mg N/L.

The data of Table 6 indicate that, as in the case with nitrogen-limited Selenastrum, phosphorus-limited Selenastrum are not provided available phosphorus by live algae. Similar evidence of this nature was noted when it was found that a phosphorus-limited Cladophora sp. (0.07 mg PO_4-P extracted/100 mg algae) grew at the bottom (3-meter depth) of Lake Mendota where it was intimately mixed with the aquatic weed,

Table 5. Live versus Killed Algae as Sources of Available Nitrogen for Selenastrum in Gorham's (-N) Medium (150 ml/Tube, pH 7).

N Source	Source of Alga	Approx. Algal Dry Weight (mg/Tube)	Growth of Selenstrum after 7-8 Days: Absorbance (1 cm, 750 mμ)	Growth of Selenstrum after 7-8 Days: Av. Absorbance per 10 mg Algae
None		--	0.02	
NO_3-N (5 mg N/L)		--	0.32	
NO_3-N (10 mg N/L)		--	0.60	
Live Algae:				
N-limited Cladophora sp.[1]	L. Mendota 6/17/69	10 40	0.03 0.02	.010
Surplus-N Cladophora sp.[2]	Monona Bay 6/17/69	10 40	0.02 0.02	.008
N_2-Fixers Anabaena sp. + Aphanizomenon sp.	L. Mendota 6/17/69	15 44	0.06 0.16	.036
Bloom Microcystis sp.	L. Monona 7/23/69	19 57	0.02 0.02	.005
Anabaena flos-aquae (Ind. 1444)	Gorham's(-N) Culture	10 40	0.02 0.03	.010
Surplus-N Selenastrum PAAP	Gorham's Culture	10 40	0.02 0.04	.012
Killed Algae (freeze-thaw):				
N-limited Cladophora sp.[1]	L. Mendota 6/17/69	10 40	0.02 0.05	.015
Surplus-N Cladophora sp.[2]	Monona Bay 6/17/69	20 40	0.14 0.15	.050
Surplus-N Cladophora sp.[3]	L. Mendota 7/25/69	20 40	0.12 0.44	.060
N_2-fixer Anabaena sp.	L. Mendota 6/19/69	10 20	0.31 0.37	0.23
Bloom Microcystis sp.	L. Monona 7/23/69	19 57	0.23 0.54	0.10
Anabaena flos-aquae (Ind. 1444)	Gorham's(-N) Culture	10 40	0.08 0.16	.050
N-limited Selenastrum	Gorham's (7.5 mg N/L)	10 40	0.01 0.01	.004
Surplus-N Selenastrum	Gorham's (82 mg N/L)	10 40	0.12 0.58	0.15

[1] N-limited Cladophora sp: 20 μg NH_3-N absorbed/10 mg/hr.

[2] Surplus-N Cladophora sp. (Monona Bay): 5 μg NH_3-N absorbed/10 mg/hr.

[3] Surplus-N Cladophora sp. (Lake Mendota): 5 μg NH_3-N absorbed/10 mg/hr.

Table 6. Live versus Killed Algae as Sources of Available Phosphorus for Selenastrum in PAAP (-P) Medium (150 ml/Tube, pH 7).

P-Source (Dialysis Tube)	Approx. Algal Dry Weight (mg/Tube)	Growth of Selenastrum after 6-9 Days	
		Absorbance (1 cm, 750 mμ)	Av. Absorbance per 10 mg Algae
None	--	0.01	
PO_4-P (0.03 mg P/Tube)	--	0.20	
Live Algae			
P-limited Cladophora sp.[1] (Monona Bay)	10	0.02	0.008
	40	0.02	
P-surplus Cladophora sp.[2] (L. Mendota)	10	0.02	.008
	40	0.02	
P-surplus Anabaena sp.(90%)[3] (L. Mendota)	10	0.07	.07
	20	0.14	
P-surplus Microcystis sp.[4] (L. Monona)	19	0.01	.003
	57	0.01	
Killed Algae (freeze-thaw)			
P-limited Cladophora sp.[1] (Monona Bay)	10	0.18	.14
	20	0.24	
P-surplus Cladophora sp.[2] (L. Mendota	5	0.32	.55
	10	0.50	
P-surplus Anabaena sp.[3] (L. Mendota)	10	0.43	.37
	20	0.70	
P-limited Aphanizomenon sp.[5] (Monona Bay)	8	0.05	.06
	23	0.14	
P-surplus Microcystis sp.[4] (L. Monona)	19	0.24	.13
	57	0.75	

[1] P-limited Cladophora sp. 0.05 mg PO_4-P extracted/100 mg algae
[2] P-surplus Cladophora sp. 0.32 mg PO_4-P " " " "
[3] P-surplus Anabaena sp. 0.23 mg PO_4-P " " " "
[4] P-surplus Microcystis sp. 0.22 mg PO_4-P " " " "
[5] P-limited Aphanizomenon sp. 0.07 mg PO_4-P " " " "

Myriophyllum sp., which contained surplus phosphorus (0.33 mg PO_4-P extracted/100 mg tip leaves) at the time of sampling (9/4/69).

The amount of growth attained by phosphorus-limited Selenastrum in the presence of killed algae was found to be dependent upon whether the phosphorus source (algae) had been itself phosphorus-limited or contained surplus phosphorus: 5 mg of Cladophora with surplus phosphorus supported more growth of Selenastrum than 20 mg of phosphorus-limited Cladophora; and 10 mg of Anabaena sp. with surplus phosphorus supported 3 times as much growth of Selenastrum as 23 mg of phosphorus-limited Aphanizomenon sp.

The data on the sharing of nutrients by algae presented thus far have only concerned the use of the green alga, Selenastrum. In order to demonstrate that similar results could be obtained with other algae, comparative tests were also carried out with the bloom-forming blue-green alga, Microcystis aeruginosa (Wis. 1036), and the N_2-fixing blue-green alga, Anabaena flos-aquae (Ind. 1444). Typical results are summarized in Table 7 as the averages of absorbances attained after 14 days of culture when different sources of nitrogen and phosphorus were evaluated.

As in previous studies, live plants provided neither nitrogen nor phosphorus to the test algae. It is of interest to point out that samples of phosphorus-limited Cladophora, even when killed, did not support the growth of the test algae, but the other plants with surplus nutrients did share available nutrients when they were killed.

Under the conditions of these tests the minimal reproducible growth of test algae that was detectable by the light absorbance measurements used would be representative of the growth attained with about 0.008 mg P or 0.06 to 0.2 mg N per culture. It is evident from the data presented that neither live algae nor live aquatic weeds did provide such quantities of available nutrients. Live algae or weeds may have provided lesser quantities of available nutrients which could not be detected by such crude techniques as used here. The facts of importance are that algae or weeds from the field or from cultures with surplus nitrogen or phosphorus do not share their nutrients unless they are killed. Therefore, once nitrogen or phosphorus are absorbed by plant materials, it must be assumed that they are not readily available in significant amounts until the death of the plant.

Table 7. Comparative Tests of Field Plants as Sources of Available Nitrogen or Phosphorus for Selenastrum, Microcystis, or Anabaena.

Medium (75 ml/125 Erl.)	Nutrient Source and Amount (mg/culture)	Av. Growth Attained after 14 Days (Absorbances, 1 cm, 750 mμ)		
		Selenastrum capricornutum (PAAP)	Microcystis aeruginosa (Wis. 1036)	Anabaena flos-aquae (Ind. 1444)
Gorham's (-P)	None	0.01	0.02	0.02
"	PO_4-P - 0.015 mg	.14	.10	.07
	Surplus-P Cladophora[1]- 20 mg			
"	Live	.01	.01	.01
"	Killed[5]	.38	.08	.24
	P-Limited Cladophora[3]- 20 mg			
"	Killed	.01	--	.02
	Myriophyllum Leaves[4]- 20 mg			
"	Live	.01	.03	.01
"	Killed	.10	.15	.12
Gorham's (-N)	None	.01	.00	--
"	NO_3-N - 0.38 mg	.24	.06	--
	Surplus-N Cladophora[2]- 20 mg			
"	Live	.02	.01	--
"	Killed	.23	.13	--

[1] Surplus-P Cladophora sp. (0.30 mg PO_4-P extracted/100 mg algae) - Lake Mendota 10/7/69
[2] Surplus-N Cladophora sp. (9 μg NH_3N utilized/10 mg/hour) - Lake Mendota 10/7/69
[3] P-Limited Cladophora sp. (0.02 mg PO_4-P extracted/100 mg) - Lake Wingra 10/7/69
[4] Myriophyllum sp. (0.05 mg PO_4-P extracted/100 mg leaves) - Lake Wingra 10/7/69
[5] Killed - plants killed by freeze-thaw (2 cycles)

DISCUSSION

As has been pointed out, tests of the availability of nutrient sources when they are in contact with algae over the entire period required for the growth of the algae (1-2 weeks) do not differentiate between the availability of the original source of the nutrient and its degradation products. Therefore, for critical analyses of the immediate availability of nutrient sources, tests with short-exposure periods are preferable. Such tests are required, of course, if one wants to evaluate nutrient sources for algae held in the dark.

The results presented here on the effects of pH and light versus darkness have indicated that algae can absorb nitrogen and phosphorus compounds under all conditions tested. The significance of these results is that pH (in a range of pH 7-9) and darkness do not affect the ability of the algae tested to absorb nitrogen and phosphorus. Therefore, nutrients could be absorbed by the algae in areas of lakes below the penetration of light. The algae then could rise or be carried to photic zones where subsequent growth could take place. The sources of nutrients available to the algae in these subsurface areas are still questionable, but the results with solubility as a factor indicate that simple solubility may not be the only reason why lake muds and natural rocks did not serve as sources of nutrients when tested in short-term experiments. It must be emphasized that the negative results with the tests of lake muds and rocks as nutrient sources reported here were carried out over fairly short periods of time (1-2 weeks). Although the laboratory results reported substantiate field tests as to the lack of available nitrogen and phosphorus from lake muds for algae in situ, it should be emphasized that studies by Lee (personal communication, 1970) have shown that very significant amounts of phosphorus are released from aerobic lake muds when tests are carried out for periods exceeding 1 month. This release is even more rapid under anaerobic conditions. Therefore, the ecological significance of the contribution of phosphorus from lake muds can not be judged properly until more studies are carried out.

An interesting point when discussing solubility as a factor in the availability of a compound is the similarity seen in nutrition and toxicity tests. It has been shown with studies of the toxicity of dichlone (2,3-dichloronaphthoquinone)[4] that 1 mg/L of the chemical does not prevent the growth of Chlorella under standard conditions, but

that 1.5 mg/L will accomplish this. Yet, the solubility of dichlone is only 0.1 mg/L, so the amounts that are insufficient and sufficient for algistatic action are both in excess of the limit of solubility. Also, it has been shown that insoluble forms of copper were as effective as algistats and algicides in algal cultures when compared with soluble forms of copper.[7] However, when copper is used as an algicide in field applications, sources of copper which remain soluble in the waters in which they are used are much more effective than the usual application of copper sulfate which readily precipitates under field conditions (R. M. Stern, 1970, personal communication). Therefore, in both toxicity and nutrition, although laboratory studies may show that insoluble sources of compounds are available to algae, under practical field conditions insoluble sources may be lost from the environment of the algae and not be effective.

The fact that live algae and aquatic weeds do not share their adequate or surplus nutrients with nutrient-limited algae, even when the same strains are used, as in the tests with Selenastrum as the source of nitrogen or phosphorus for nutrient-limited Selenastrum, has considerable ecological importance. It can be concluded that once nitrogen and phosphorus are tied up in living plant material those nutrients are not available for the nutrition of other plants until the original plants die. Therefore, once lake waters are stripped of available nutrients by plant production, further plant production will depend upon nutrients from continuous sources of nutrients, such as wastewater effluents, the regeneration of nutrients from muds, or the decay of the original flora. Knowledge of the relative rate of supply from each source of nutrients could be of great value in evaluating the influence of any potential nutrient source to a lake environment.

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ACETYLENE REDUCTION ASSAY FOR DETERMINATION OF PHOSPHORUS AVAILABILITY IN WISCONSIN LAKES

W. D. P. Stewart, G. P. Fitzgerald,
and R. H. Burris

ABSTRACT

Phosphorus-starved cells of Anabaena flos-aquae rapidly increase their capacity to reduce acetylene to ethylene when they receive phosphorus. This response may be used as a bioassay for detecting available phosphorus in aquatic ecosystems. The sensitivity of the method compares favorably with conventional methods for measuring dissolved orthophosphate, and has the additional advantage that it measures available phosphorus. Studies on Wisconsin lakes show that available phosphorus generally is present, that the concentrations are higher at the lower depths than at the surface, and that there may be diurnal variations in the available phosphorus content of surface waters. Important sources of available phosphorus in Lake Mendota are the waters below the thermocline and the input from storm sewers.

INTRODUCTION

Eutrophication of aquatic ecosystems has become intensified in many areas within recent years and it is considered generally that nitrogen and phosphorus are the most important nutrients that contribute to eutrophication.[1-4] However, Stewart, Fitzgerald, and Burris[5,6] found, using the acetylene reduction technique[7,8] in lakes, that when metabolically active heterocystous blue-green algae were present they were invariably capable of fixing N_2. This infers that even if it were possible to eliminate completely the input of combined nitrogen into lakes, blooms of nitrogen-fixing algae would still occur if phosphorus was available. Thus it is important to know the quantities of available phosphorus in, and being added to, lakes, and to date there has been no completely satisfactory method[9,10] whereby this may be determined.

This paper reports a modification of the acetylene reduction technique which may allow rapid and sensitive determinations of the available phosphorus in aquatic ecosystems. The bioassay is based on the following rationale: If a culture of a nitrogen-fixing blue-green alga is grown on molecular

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nitrogen under conditions of phosphorus starvation, the organism should respond to the addition of available phosphorus with increased growth and metabolism. Because the organism fixes N_2, the enhancement of metabolism when phosphorus is supplied should be reflected in an increased capacity for the reduction of acetylene to ethylene.

RESULTS

Experiments Using a Defined Medium

Experiments were conducted to determine the response of blue-green algae to various nutrients. The algae were grown in Gorham's complete nitrogen-free medium and were transferred after washing into a medium in which one of phosphorus, iron, magnesium, or sulfur was omitted. When the algae showed nutrient deficiency they were returned to complete Gorham's nitrogen-free medium. The data in Table 1 show that within 2.0 hr nutrient-depleted algae responded to phosphorus but not to the other elements. A longer incubation may have elicited a response to the other nutrients, but a long incubation period defeats the object of a rapid bioassay technique. Nevertheless, bioassays for other elements based on the acetylene reduction assay may be reasonable, when it is appreciated that existing bioassay methods[9,10] require 10-14 days.

The response to phosphorus depends upon the test alga being starved initially as is shown in Table 2. Natural populations of algae could not be used satisfactorily as assay material either (Table 3), because sometimes a response was obtained and sometimes it was not. This irregularity probably reflects the ability of algae to store phosphorus which can be utilized later in phosphorus-deficient waters.

The data in Table 4 show that phosphorus-starved algae respond to as little as 3.0 µg phosphorus/liter, but that high concentrations saturate the system. Addition of the same amount of phosphorus may induce different enhancements in acetylene reduction in different experiments and this usually reflects different degrees of phosphorus starvation in the test algae used. The type of response shown in Figure 1 is characteristic for phosphorus levels in the range 0-0.25 mg/liter.

The time course of the response (Fig. 2) shows a lag phase of almost 15 min followed by a 15-min period of rapid response. After 30 min there was little further increase in the rate of acetylene reduction. The positive value obtained over the control at 0 time presumably resulted from phosphorus uptake during the period in which the algae were being separated from the phosphorus-containing medium by centrifugation immediately after the incubation period and prior to measurement of acetylene

Table 1. Response in Acetylene Reduction of Nutrient-Starved Anabaena flos-aquae to Various Nutrients.

Deficient Nutrient	Test Medium	nmol C_2H_4/ 1.0 ml/min
Sulfur	- sulfur	0.057
	+ sulfur	0.064
Iron	- iron	0.228
	+ iron	0.212
Magnesium	- magnesium	0.465
	+ magnesium	0.452
Phosphorus	- phosphorus	0.027
	+ phosphorus	0.349

Incubation period prior to the 30 min acetylene reduction assay was 2 hr; the volume of test water used was 70 ml, the nutrient concentrations in complete medium were (mg/liter): S,10; P, 7; Mg, 1; Fe, 0.05. Each value is the mean of triplicate determinations.

Table 2. Acetylene Reduction by Phosphorus-Starved and Phosphorus-Sufficient Anabaena flos-aquae When Incubated in Phosphorus-Containing Medium.

Pretreatment	Subsequent Treatment	nmol C_2H_4/ 1.0 ml/min
+ phosphorus	- phosphorus	0.406
	+ phosphorus	0.392
- phosphorus	- phosphorus	0.030
	+ phosphorus	0.349

Pretreatment period was 4 days; the subsequent incubation period prior to the 30 min C_2H_2 reduction assay was 2 hr; the volume of solution used was 70 ml. Each value is the mean of triplicate determinations.

Table 3. Response of Natural Populations of Anabaena and of a Phosphorus-Starved Laboratory Culture of A. flos-aquae when Incubated in the Surface Waters of Lake Mendota, with or without a Phosphorus Supplement.

Date	Alga Used	Treatment (mg P/Liter)	nmol C_2H_4/ 1.0 ml/min
8/21/1969	Natural Bloom of Anabaena	Untreated	0.23
		+0.085	1.94
		+0.143	2.01
9/3/1969	Natural Bloom of Anabaena	Untreated	0.42
		+0.085	0.40
		+0.143	0.41
9/3/1969	P-Starved Laboratory Culture of Anabaena flos-aquae	Untreated	0.02
		+0.085	0.23
		+0.143	0.20

Period of incubation prior to the C_2H_2 reduction assay was 2 hr; volume of water used was 35 ml. In the experiments of Sept. 3rd the natural populations of Anabaena and of the P-starved A. flos-aquae were incubated in aliquots of the same test water. Date of collection of water samples is given in column 1.

Table 4. Response in Acetylene Reduction of Phosphorus-Starved A. flos-aquae Cultures to Various Levels of Available Phosphorus.

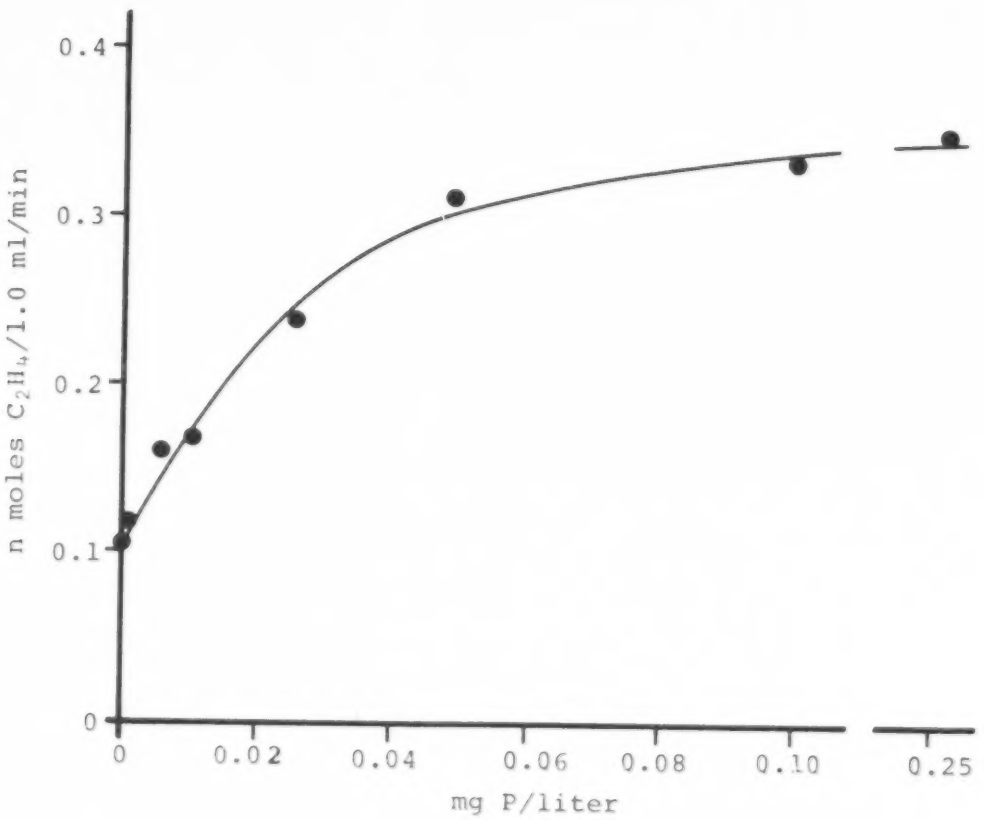
Phosphorus Added (mg/Liter)	nmol C_2H_4/ 1.0 ml/min	Increase Over P-Deficient Cultures (%)
	Experiment 1	
0.000	0.010	-
5.700	0.118	1080
1.430	0.119	1090
0.360	0.125	1150
0.087	0.119	1090
0.014	0.048	380
	Experiment 2	
0.000	0.036	-
0.010	0.116	222
0.005	0.066	83
0.003	0.042	17
0.001	0.036	0

Expt. 1: Incubation period prior to the 30 min C_2H_2 reduction assay was 60 min; the volume of the test water used was 25 ml.

Expt. 2: Incubation period was 60 min and the volume of test water used was 500 ml.

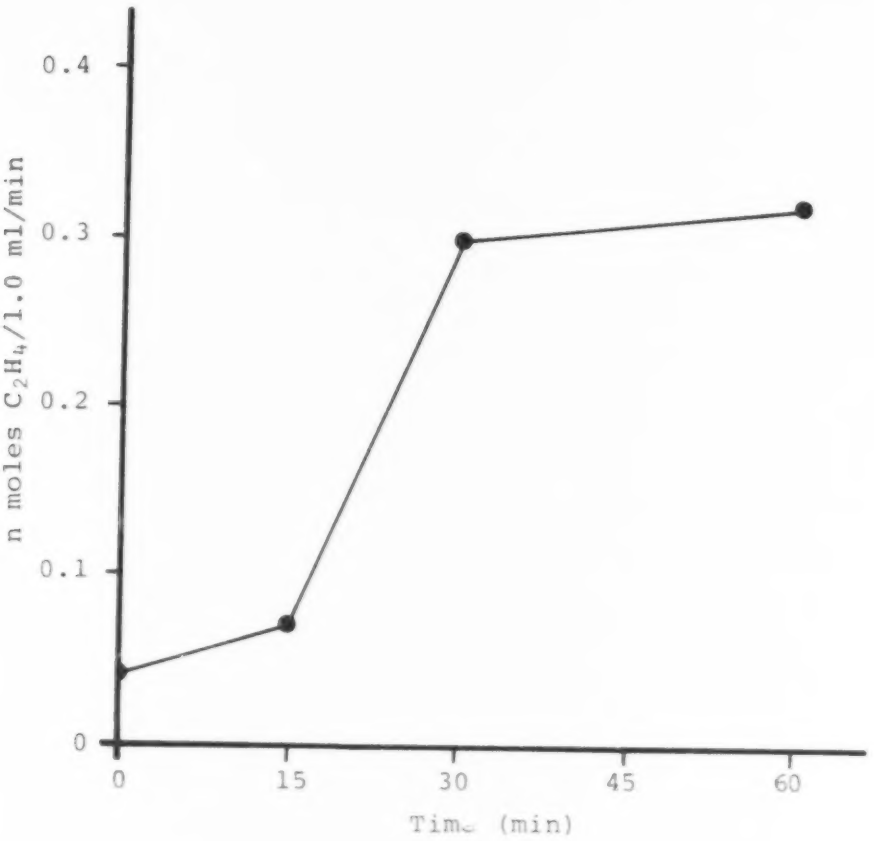
Each value is the mean of triplicate determinations.

Figure 1. Response of P-Starved A. flos-aquae to Various Levels of Available P.



The incubation period prior to the 30 min C_2H_2 reduction assay was 60 min; the volumes of the test solutions used were 100 ml. Each value is the mean of triplicate determinations.

Figure 2. Effect of Time on the Response to Phosphorus of Phosphorus-Deficient Anabaena Cultures



The incubation period prior to the 30 min C_2H_2 reduction assay was in the presence of 0.02 mg P/liter for the time indicated on the figure; the volume of test solution used was 70 ml. Each value is the mean of triplicate determinations.

reduction. A 30-min incubation in the test water before the start of the acetylene reduction assay is probably sufficient enough to provide reliable results.

Field Trials

The data in Table 5 show that phosphorus-starved Anabaena responded to incubation in the surface waters of Lake Mendota, Lake Wingra, and Lake Monona, indicating that each contained available phosphorus with the highest levels occurring in Lake Mendota. Table 6 shows that available phosphorus is highest in Lake Mendota in the morning and decreases during the day. Thus, available phosphorus levels may build up in lakes at night and decrease during the day when vigorous algal metabolism occurs as a result of photosynthesis. Others[13,14] have also reported a more rapid uptake of phosphorus in the light than in the dark. More experiments are required to determine whether the variations which we detected are characteristic of the surface waters of Lake Mendota.

Variations in the levels of available phosphorus at various depths of Lake Mendota were examined. Table 7 shows that the assay organism responded markedly when placed in untreated waters from all depths with the greatest response being obtained in waters from 15 m and 18 m; the lake was 18.5 m deep at the sampling site studied and the thermocline was located near 10-12 m. When phosphorus was added to these waters, there was a marked improvement in the nutrient quality of the upper waters. In contrast there was less response to added phosphorus in the waters from 18 m and particularly from 15 m. These results suggest that phosphorus was limiting particularly in the surface waters and that at the lower depths, although more available phosphorus was present, there was also present an inhibitory factor of some type.

A practical application of the bioassay is illustrated with data obtained on waters of University Bay Creek, which discharges storm sewer drainage from urban areas of Madison into Lake Mendota. Table 8 shows that the phosphorus-limited test algae responded markedly when placed in these waters. The response persisted in lake water near the inlet of the creek, but the response decreased as the water was diluted in the main part of the lake. It is apparent that the runoff from urban areas may drastically influence the nutrient balance of Lake Mendota.

Table 5. Response in Acetylene Reduction of Phosphorus-Starved A. flos-aquae when Incubated in the Surface Waters of Various Lakes.

Sample	Treatment	nmol C_2H_4/ 1.0 ml/min	Increase on Adding P (%)
Lake Mendota	Untreated	0.12	-
	+ Phosphorus	0.16	33
Lake Monona	Untreated	0.04	-
	+ Phosphorus	0.11	175
Lake Wingra	Untreated	0.08	-
	+ Phosphorus	0.33	313

Period of incubation prior to the C_2H_2 reduction assay was 60 min; volume of water used was 70 ml; samples collected on Aug. 6, 1969; phosphorus was added as 1.0 mg K_2HPO_4/liter. Each value is the mean of triplicate determinations.

Table 6. Response of Phosphorus-Starved A. flos-aquae when Placed in the Surface Waters of Lake Mendota Collected at Various Times of the Day.

Sampling Time	nmol C_2H_4/ 1.0 ml/min
7 a.m.	0.07
12 noon	0.02
4 p.m.	0.01
8 p.m.	0.01

Incubation period prior to the 30 min C_2H_2 reduction assay was 60 min; volume of test water used was 200 ml. Samples collected on Sept. 9, 1969. Each value is the mean of triplicate determinations. The times given are central standard time.

Table 7. Acetylene Reduction by Phosphorus-Starved A. flos-aquae when Incubated in Untreated Waters and in Phosphorus-Supplemented Waters Taken from Various Depths of Lake Mendota.

Depth (m)	nmol C_2H_4/1.0 ml/min	
	Untreated Lake Water	Lake Water + 0.01 mg P/Liter
0	0.04	0.43
2	0.07	0.38
8	0.06	0.38
10	0.06	0.34
15	0.16	0.15
18	0.16	0.23

Period of incubation prior to the 30 min C_2H_2 reduction assay was 75 min; volume of lake water used was 200 ml; value for the control medium -P was 0.02 nmol C_2H_4/1.0 ml/min. Samples collected on Sept. 12, 1969. Each value is the mean of triplicate determinations.

Table 8. Acetylene Reduction by Phosphorus-Deficient A. flos-aquae Incubated in Lake Mendota Surface Waters Taken near a Drainage Inlet.

Test Water	nmol C_2H_4/ 1.0 ml/min
University Bay Creek	0.42
University Bay at Inlet of Creek	0.42
25 m Offshore from Inlet	0.14
250 m Offshore from Inlet	0.06
1000 m Offshore from Inlet	0.04

University Bay Creek water was sampled at a point just before it entered the lake. The period of incubation prior to the C_2H_2 reduction assay was 60 min; volume of lake water used was 200 ml; samples collected on Sept. 10, 1969; value for the control medium -P was 0.03 nmol C_2H_4/1.0 ml/min. Each value is the mean of triplicate determinations.

DISCUSSION

Two main methods of measuring phosphorus in aquatic ecosystems are currently employed. The most common is to determine chemically the levels of orthophosphate. This method takes no account, however, of other important types of phosphorus which may be present. For example, Galloway and Krauss[15] showed that inorganic polyphosphates could be utilized by Chlorella at comparable rates to orthophosphate, and substances such as glucose-6-PO_4 may be utilized by marine algae.[16] The other method is to measure the extractable phosphorus present in algae and to correlate this with the amount of alkaline phosphatase activity of the algae.[12] The latter method, though useful, has the disadvantage that the algae rapidly assimilate and store phosphorus when it is available; thus, the phosphorus content of an alga is no real indication of the available phosphorus present in a particular water at a particular time.

The present results, although preliminary, indicate that a bioassay method based on the acetylene reduction technique may be practical and have advantages over previously available methods. The method which we suggest is basically that presented in the Methods section, but the following additional points are relevant.

Anabaena flos-aquae is a good assay organism, but any fast-growing N_2-fixing blue-green alga presumably could be used. The exact culture medium used is also immaterial as long as it is free of combined nitrogen and the bioassay organism is phosphorus starved and metabolically active at the start of the assay.

Small volumes (25 ml) of test water may be used, but larger volumes are more satisfactory. A volume of 70-200 ml of water is recommended with 6.0 mg dry weight of the assay organism. The volume and amount of algae should be constant in any one test series.

The data in Figure 2 suggest that an incubation period of 30 min with constant shaking is adequate. The algal suspension is then concentrated by centrifugation to a volume of 3.0 ml and three 1.0-ml aliquots are then incubated under acetylene for 30 min.

All test samples which are being compared should be set up at the same time. For each test water sample
(a) there should also be available a control sample

(b) in which the algal response in phosphorus-free medium is noted, and a second control (c) comprising the test water sample plus 0.025 mg/liter of phosphorus. The value for (b) serves to show that the bioassay organism is phosphorus starved while the value for (c) shows its ability to respond in the test water when phosphorus is available. The relative response for each test water thus will be: (c - b)/(a - b). By comparing the values for this ratio, the relative abundance of phosphorus in each test sample can be measured.

The quantitative determination of the amount of available phosphorus in a particular water is more complicated, and it is suggested that the following series should be set up for such a measurement: (1) P-free medium, (2) P-free medium + 0.100 mg P/liter, (3) P-free medium + 0.050 mg P/liter, (4) P-free medium + 0.025 mg P/liter, (5) P-free medium + 0.010 mg P/liter, (6) test water sample + 0.025 mg P/liter.

Tests (1)-(5) show the response of the alga to available phosphorus in the absence of inhibitory or competitive reactions and can be used to prepare a standard curve which can be used for all tests performed with the same batch of assay organism. The value for test (7) minus test (1) gives the response to 0.025 mg/liter of phosphorus plus the amount of phosphorus in the water sample. The value for test (6) minus test (1) gives the response to available phosphorus in the test water only. Thus, the response to 0.025 mg P/liter alone and to the available phosphorus in the test water is obtained. These values are then transferred onto the standard curve to give the quantity of available phosphorus in the test water. Thus, the response of the algae to a series of standards and to two samples of the test water, one with and the other without added phosphorus, is all that is required to determine the level of available phosphorus in a particular water. The same standard curve can be used for all waters tested using the same batch of assay organism. We always have noted a response when phosphorus was added to phosphorus-deficient culture medium. This indicates that a failure to detect acetylene reduction in test water samples with added phosphorus is because of some inhibitory factor in the water. In the presence of total inhibitors the test would not be satisfactory.

The overall sensitivity of the phosphorus bioassay is good. For example, the stannous chloride method recommended by the American Public Health Association (1965)[17] detects orthophosphate-phosphorus levels as low as 10 µg/liter,

whereas the acetylene bioassay technique is probably more sensitive and detects available, rather than total, phosphorus. Also, biological nitrogen fixation and phosphorus availability can be studied with similar equipment.

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27 Abstract

Critical evaluations are presented of bioassays for nutrient availability. The biological availability of any required algal nutrient in a sample of water can be determined by growth experiments requiring 2 weeks incubation. In addition, relatively short-term tests can be carried out measuring changes in certain enzymatic activities or chemical fractions which have been shown to reflect meaningful nutritional changes. The latter types of tests have been useful in evaluating the nutritional status of in situ algae. The selection of the type of bioassays for particular purposes can be made from the data presented as to what information can be obtained, the length of time required, and the range of sensitivity of the bioassays. Examples are presented of ecologically important questions which have been answered by the different bioassays.

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